REMARKS

Claim amendments

Claims 8, 16, 20 and 24 have been amended to an independent format. Claims 30-45 have been added. Support for new Claims 30-45 can be found, for example, in original Claims 2-6.

Objection to Claims 8, 16, 20 and 24

Claims 8, 16, 20 and 24 are objected to because they depend on non-elected Claims 1, 9, 17 and 21, respectively.

Claims 8, 16, 20 and 24 have been rewritten in independent format, thereby obviating the objection.

Rejection of Claims 8, 16, 20 and 24 under 35 U.S.C. §112, first paragraph

Claims 8, 16, 20 and 24 are rejected under 35 U.S.C. §112, first paragraph "as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention" (Office Action, page 3). The Examiner states that the "specification fails to provide adequate guidance and evidence for whether sufficient polynucleotide or vector encoding IL-12 protein would be present in target site of the host such that sufficient IL-12 protein is obtained to induce or enhance immune response to a TI antigen in a host, or to induce an immune response to *Streptococcus pneumoniae* or *Neisseria meningiditis* in a host via various administration routes of said polynucleotide" (Office Action, page 4). The Examiner further states that the "claims read on gene therapy" and that the "state of the art for gene therapy was unpredictable at the time of the invention", citing the Deonarian, Eck *et al.* and Gorecki *et al.* references in support thereof (Office Action, page 5). The Examiner concludes that "it would have required undue experimentation for one skilled in the art at the time of the invention to practice over the full scope of the invention claimed" (Office Action, page 6).

Applicants respectfully disagree. The first paragraph of § 112 requires nothing more than objective enablement (*In re Marzocchi & Horton* 169 USPQ 367, 369 (CCPA 1971)). In *Marzocchi* the court stated that:

> a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of § 112 *unless* there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. Assuming that sufficient reason for such doubt does exist, a rejection for failure to teach how to make and/or use will be proper on that basis; such a rejection can be overcome by suitable proofs indicating that the teaching contained in the specification is truly enabling. Id.

The court further stated that:

> it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Id. at 370.

The Examiner has not provided acceptable evidence or reasoning which would cast doubt on Applicants' claimed method of inducing or enhancing an immune response to a T-cell independent antigen in a host, which comprises administering to the host an effective amount of interleukin-12 and the T-cell independent antigen, wherein the interleukin-12 is administered as a polynucleotide under conditions in which the interleukin-12 is expressed *in vivo* and an immune response to the T-cell independent antigen is induced or enhanced in the host.

In the specification as filed, Applicants demonstrate that administration of IL-12 diluted in PBS and a TI antigen (*i.e.,* DNP; A, C, Y and W-135 capsular serogroups of the meningococcal vaccine; purified capsular polysaccharides from 23 serotypes of *S. pneumoniae* of the pneumococcal vaccine) induce or enhance an immune response to the antigen *in vivo* (specification, page 13, line 25 - page 21, line 10). Applicants further teach that "the IL-12 and/or TI antigen can be administered by *in vivo* expression of polynucleotides" and provide adequate guidance to one of skill in the art for doing so (specification, page 8, lines 4-5). Specifically, Applicants teach that:

> Several expression vector systems are available commercially or can be reproduced according to recombinant DNA and cell culture techniques. For example, ***vector systems*** such as the yeast or vaccinia virus expression systems, or virus vectors can be used in the methods and compositions of the present invention . . . Other techniques using ***naked plasmids or DNA***, and ***cloned genes***

> ***encapsulated in targeted liposomes or in erythrocyte ghosts***, can be used to introduce IL-12 and/or TI antigen polynucleotides into the host . . . The constructions of expression vectors and the transfer of vectors and nucleic acids into various host cells can be accomplished using genetic engineering techniques, as described in manuals like *Molecular Cloning* and *Current Protocols in Molecular Biology*, which are hereby incorporated by reference, or by using commercially available kits (Sambrook, J., *et al., Molecular Cloning*, Cold Spring Harbor Press, 1989; Ausubel, F.M., *et al., Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley-Interscience, 1989) (specification, page 8, lines 17-31, emphasis added)

Thus, in the specification as filed, Applicants have provided sufficient guidance that enables one of skill in the art to practice the invention without undue experimentation.

Furthermore, the specification need not disclose what is well-known to those skilled in the art and preferably omits that which is well-known to those skilled and already available to the public (*In re Buchner*, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991)). At the time of Applicants' invention, methods of administering and expressing the IL-12 gene *in vivo*, and that such methods could be used in gene based therapies, were known to those of skill in the art.

For example, Applicants direct the Examiner's attention to Tahara, H., *et al., J. Immunol., 154:*6466-6474 (1995) which is being filed as Exhibit A. Tahara *et al.* show eradication of murine tumors with IL-12 gene therapy using a polycistronic retroviral vector and conclude that their "results support the ***feasibility of IL-12 gene therapy*** for the treatment of human cancer" (Tahara *et al.*, abstract, emphasis added). Rakhmilevich, A.L., *et al., Proc. Natl. Acad. Sci., USA, 93*:6291-6296 (1996) which is being filed as Exhibit B, "evaluated the effect of ***IL-12 gene therapy*** on the growth of established tumors" and show that gene gun-mediated administration of the IL-12 gene results in regression of established primary and metastatic tumors (Rakhmilevich *et al.*, page 6291, column 2, emphasis added). Kim *et al.* show that administration of an IL-12 expression vector along with a DNA vaccine cassette for HIV-1 significantly enhanced "CTL response in vivo" with four different HIV-1 DNA immunogens (Kim, J.J. *et al. J. Immunol., 158:*816-826 (1997) which is being filed as Exhibit C). Kim *et al.* conclude that their work "demonstrates the power of DNA delivery in vivo for both the production of a new generation of more effective and targeted vaccines or immunotherapies" (Kim *et al.*, abstract). Jiang *et al.* engineered a retroviral construct containing the cDNA encoding the p40 and p35 subunits of IL-

12 as a single chain" and that treatment of mice with macrophages that have been transduced with the single chain IL-12 retroviral construct "afforded a single level of protection against lethal pulmonary challenge" with the fungal pathogen, *Coccidioides immiti* (Jiang, C., *et al., Infect. Immun., 67(6):*2996-3001 (1999) which is being filed as Exhibit D). Watanabe *et al.* show that *in vivo* administration of ***IL-12 naked DNA*** induce "IL-12 mediated biological activity" and conclude that "***this form of gene therapy is efficient and safe for IL-12 delivery***" (Watanabe, M., *et al., J. Immunol., 163:*1943-1950 (1999), page 1944, column 1, emphasis added which is being filed as Exhibit E).

Clearly, Applicants provide adequate guidance and evidence for determining whether a polynucleotide or vector encoding IL-12 protein would be present in target site of the host such that sufficient IL-12 protein is obtained to induce or enhance immune response to a TI antigen in a host. In addition, it is clear that the state of the art for IL-12-based gene therapy (*e.g.,* Exhibits A-E) was not unpredictable at the time of Applicants' invention, and undue experimentation is not required for one skilled in the art to practice the full scope of Applicants' claimed invention.

None of the references the Examiner has cited in support of the enablement rejection discussed IL-12 based gene therapy, and thus, none of the references is relevant to Applicants' claimed invention.

Deonarian notes the drawbacks of viral methods for gene delivery, however, most methods of therapy have drawbacks which can be addressed. The Examiner states that Deonarian teaches that "one of the biggest problems hampering successful gene therapy is the ability to target a gene to a significant population of cells and express it at adequate levels for a long enough period of time'" (Office Action, page 4). Such problems can be addressed, for example, by administering multiple doses. In addition, as Eck *et al.* note, "long-term gene expression (months to years)" . . . is likely necessary to treat genetic diseases" (Eck, *et al.*, page 78, column 2), which is not the subject of Applicants' claimed invention; and as Gorecki notes "[t]o treat some disorders (e.g., genetic defects) long-term expression would be required while for others (e.g., cancer) a transient burst of expression may be sufficient" (Gorecki, page 189, column 1). Furthermore, as Deonarian notes, improved viral vectors are being developed and there is "a growing body of research" in non-infectious gene delivery methods (*e.g.,* liposomes, naked DNA, ligand-targeted receptor-mediated endocytosis). Specifically, Deonarian reviews

ligand-targeted receptor-mediated endocytosis (RME) which utilize synthetic complexes composed of a cell-specific targeting ligand, coupled to a DNA binding element and endosmolytic function" (Deonarian, abstract). Deonarian clearly states that there are "now many ligand/receptor systems under investigation, each one demonstrating ***successful gene transfer*** with a higher level of specificity than viruses can offer" (Deonarian, abstract, emphasis added).

The Examiner states that Eck *et al.* teach that "the fate of the DNA vector itself . . , the *in vivo* consequences of altered gene expression and protein function, the fraction of vector taken up by the target cell population, the trafficking of the genetic material within cellular organelles, and the rate of degradation of the DNA, the level of mRNA produced, the stability of the mRNA produced, the amount and stability of the protein produced, and the protein's compartmentalization within the cell, or its secretory fate, once produced are all important factors for a successful gene therapy" (Office Action, pages 4-5).

Applicants respectfully disagree. Eck *et al.* teach that "[i]t is conceivable, although yet to be realized, that each of these events ***may be incorporated*** into the design of the gene transfer system in a rational way so as to tailor gene transfer to the specific requirements of the disease being treated" (Eck *et al.,* page 82, column 1). Eck *et al.* teach that the "ideal" DNA delivery system "does not exist" (Eck *et al.*, page 83, column 1). Indeed, there are few, if any, "ideal" methods of therapy, however, less than ideal methods of therapy are still used in methods of treatment. Eck *et al.* states that "[a]s of 1995, three gene transfer systems (retroviral vectors, adenoviral vectors, and liposomes) ***has been used in human gene therapy trials***" and further discuss "conceptual strategies and issues to be refined" for various types of gene therapy *e.g.,* organ-directed gene therapy, cancer gene therapy, gene transfer into hematopoietic stem cells and gene therapy for infectious disease (Eck *et al.*, page 82, column 1; Table 5-1). Eck *et al.* do not teach, however, that undue experimentation would be required to practice Applicants' claimed method of inducing or enhancing an immune response to a T-cell independent antigen in a host, which comprises administering to the host an effective amount of interleukin-12 and the T-cell independent antigen, wherein the interleukin-12 is administered as a polynucleotide under conditions in which the interleukin-12 is expressed *in vivo* and an immune response to the T-cell independent antigen is induced or enhanced in the host.

Similar to Eck *et al.*, Gorecki *et al.* provide an update of prospects and problems of various gene therapy methods and note that we have not yet created the "optimal vector" (Gorecki, page 194, column 1). Gorecki *et al.* do not teach, however, that undue experimentation would be required to practice Applicants' claimed invention.

Clearly, the art cited by the Examiner in support of the enablement rejection does not cast doubt on Applicants' claimed method of inducing or enhancing an immune response to a T-cell independent antigen in a host, which comprises administering to the host an effective amount of interleukin-12 and the T-cell independent antigen, wherein the interleukin-12 is administered as a polynucleotide under conditions in which the interleukin-12 is expressed *in vivo* and an immune response to the T-cell independent antigen is induced or enhanced in the host. In fact, at the time of Applicants' invention, the state of the art was such that undue experimentation was not required to administer and express the IL-12 gene, and produce a therapeutic effect *in vivo*.

Applicants have provided an enabling disclosure for the full scope of the claimed invention. Reconsideration and withdrawal of the rejection is respectfully requested.

CONCLUSION

In view of the above amendments and remarks, it is believed that all claims are in condition for allowance, and it is respectfully requested that the application be passed to issue. If the Examiner feels that a telephone conference would expedite prosecution of this case, the Examiner is invited to call the undersigned.

Respectfully submitted,
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Effective Eradication of Established Murine Tumors with IL-12 Gene Therapy Using a Polycistronic Retroviral Vector[1]

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Our recent studies using IL-12 protein or fibroblasts genetically engineered to secrete IL-12 have demonstrated profound antitumor effects of IL-12 in murine models. The antitumor effects of local, high level IL-12 expression were examined using a retroviral vector, which can express both IL-12 subunits (p35 and p40) and the neomycin phosphotransferase (*Neo*)-marker gene from a polycistronic message utilizing internal ribosome entry site sequences. All animals intradermally (i.d.) receiving MCA207 murine sarcoma cell line nontransfected or *Neo*-transfected had progressively growing tumor, whereas all animals injected with MCA207 transfected with IL-12 were tumor free and were subsequently determined to be immune to a rechallenge of nontransfected MCA207 i.d. Similar results were obtained in experiments using the poorly immunogenic MCA102 murine sarcoma cell line. The inoculation of live MCA207-IL-12 tumor cells also caused the regression of contralateral nontransfected MCA207 inoculated either at the same time (80% protection) or up to 3 days before (33% protection) to the therapeutic tumor inoculation. In vivo depletion studies suggest that NK cells and IFN-γ play important roles in the development of the early phase of the antitumor response, but that T cells (both $CD4^+$ and $CD8^+$) play the major role in the subsequent events, leading to long-term immunity. The potent antitumor effects observed for paracrine gene-delivered administration of IL-12 have thus been confirmed for multiple tumor cell types and in multiple murine strains. We believe that these results support the feasibility of IL-12 gene therapy for the treatment of human cancer. *The Journal of Immunology,* 1995, 154: 6466–6474.

IL-12 exerts a variety of biologic effects on human T and NK cells in vitro (1–7). Its ability to stimulate directly the production of IFN-γ both in vitro (4, 5) and in vivo (8) and to induce primarily a Th1 (cellular immune) response in vitro (7) suggests its potential utility as an antitumor agent. Indeed, recent studies implementing systemic administration of the rIL-12 protein by Brunda et al. (9) as well as by our group (10) revealed that IL-12 has profound antitumor effects in virtually every murine tumor model evaluated. These studies have demonstrated that systemic administration of IL-12 can be initiated as late as day 28 after injection of the M5076 reticulum cell sarcoma, resulting in inhibition of tumor growth, reduction in the number of metastases, and an increase in survival time. Although IL-12 has demonstrated potent antitumor effects when injected systemically, systemic adverse effects can be observed in some strains of mice (i.e., C3H mice) (Michael Brunda, unpublished observations) and in primates. Induction of long-term immunity is less frequent in these murine models and variable from experiment to experiment (9, 10). Interestingly, Brunda et al. observed that the best therapeutic results with systemic IL-12 administration (complete regression of the tumor and induction of protective immunity against tumor rechallenge) were observed after peri-tumoral injections of IL-12 in a s.c. tumor model using Renca cells (9). These findings suggested that administration of IL-12 at the site of tumor might approximate the natural immune response. We have recently reported the antitumor effects of IL-12 in an animal model

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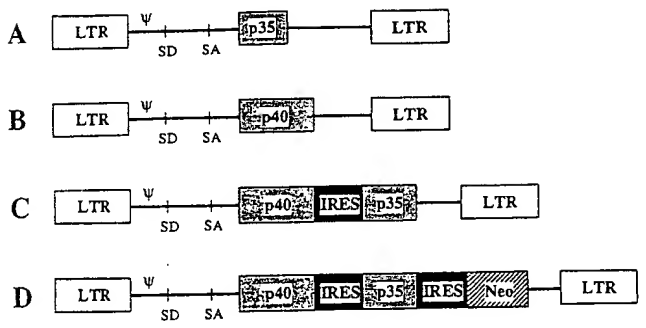

FIGURE 1. Structure of retroviral constructs containing cDNAs of mIL-12. (for details, see *Materials and Methods*). Single gene (MFG), double genes (DFG), or triple genes (TFG) were inserted into the retroviral backbone (MFG).

using fibroblasts (NIH3T3 cells) genetically engineered to secrete murine IL-12 (mIL-12)[3] (11). Local delivery of IL-12 in this model suppressed tumor growth in a dose-dependent manner and was associated with the development of an antitumor immune response.

Because the NIH3T3 cells that we used to continuously deliver IL-12 at the site of the tumor are allogeneic to the C57BL/6 mice that we used, development of a local allogeneic immune response complicated interpretation of our results. Furthermore, the expression level of IL-12 secreted by transfected NIH3T3 cells was relatively low. Thus the development of efficient expression vectors allowing for the transfection of IL-12 genes into autologous tumor cells or fibroblasts was critical to evaluate the efficacies of paracrine delivery of IL-12 and to also ultimately apply IL-12 gene therapy clinically.

A major obstacle for developing efficient retroviral delivery of IL-12 results from the heterodimeric structure of IL-12. This cytokine consists of a disulfide-linked 35-kDa light chain (p35) and 40-kDa heavy chain (p40) (1, 2). Simultaneous transfection of mammalian cells with two different genes is thus necessary for production of biologically active IL-12, unlike other cytokines used in previously reported gene therapy approaches (12, 13). Thus we initiated the present study by developing a single retroviral vector that allows us to express coordinately the genes encoding both subunits of mIL-12 at a high level along with a third gene encoding the selectable marker, neomycin phosphotransferase. The existing strategies for expression of multiple genes using regulated splicing or introducing exogeneous promoters raised substantial concern about discordant production of the two chains from transfected cells. This is a potential problem because excess p40 may inhibit the broader effects of the IL-12 heterodimer in mice (14). To overcome this problem, we developed vectors using an internal ribosome entry site (IRES) sequence which was obtained from the 5′ nontranslated region of encephalomyocarditis virus (EMCV), and allows cap-independent translation (15, 16). A single polycistronic transcript from two separate genes can thus be transcribed after the retroviral LTR and a second and third gene can be translated in a cap-independent manner. Using this retroviral vector system, we have examined the antitumor effects of IL-12 paracrine secretion on various murine tumors in vivo and have investigated the immune regulatory mechanisms responsible for IL-12-induced antitumor activity.

[3] Abbreviations used in this paper: mIL-12, murine IL-12; IRES, internal ribosome entry site; EMCV, encephalomyocarditis virus; i.d., intradermal, intradermally; ASGM1, antiasialo GM1 antiserum; hIL-12, human IL-12.

Materials and Methods

Recombinant retroviral vectors

The cDNAs of p35 and p40 of mIL-12 or previously described (11) were subcloned into MFG (17) (Fig. 1, *A* and *B*). A modified MFG-based retroviral vector termed DFG-mIL-12 (Fig. 1*C*) was constructed using a fragment of the MFG-p35 construct (*Nco*I to *Hin*dIII) and one from the MFG-p40 construct (*Hin*dIII to *Bam*HI) with an intervening IRES fragment of equine EMCV obtained from the pCITE plasmid (Novagen, Madison, WI). To obtain a retrovirus that carries both of the mIL-12 genes as well as the neomycin phosphotranspherase gene (*Neo*) as a selectable marker, an IRES-*Neo* cassette, consisting of an EMCV IRES sequence and cDNA of *Neo* obtained from pMC1neo poly(A) (Stratagene, La Jolla, CA), was subcloned into the *Bam*HI site at the 3′ terminus of the p35 cDNA in DFG-mIL-12. This vector, capable of coordinately expressing three independent genes, was termed TFG-mIL-12-*Neo* (see Fig. 3*D*). Retroviral supernatant was generated by transfecting DFG-mIL-12 or TFG-mIL-12-*Neo* proviral constructs into the CRIP packaging cell line (18) or the BOSC23 packaging cell line (19) (generously provided by Drs. Pear and Baltimore, The Rockfeller University, New York, NY). The titer of the retroviral supernatant used in the subsequent experiments was 5×10^4 to 5×10^5 CFU/ml. Absence of replication-competent virus in the cytokine-producing cells was confirmed by using a BAG mobilization assay. Target cells were infected with 2 ml of DFG-mIL-12 or TFG-mIL-12-*Neo* retroviral supernatant in the presence of polybrene (8 μg/ml). IL-12 expression level from DFG-mIL-12-infected cells was measured after one passage. When using TFG-mIL-12-*Neo*, infected cells were subsequently selected in complete medium with G418 at a concentration of 0.75 mg/ml for more than 2 wk. G418-resistant colonies were subsequently collected and used for measurement of in vitro expression or in vivo experiments. To compare the expression of cells infected with each of the mIL-12 retroviral vectors, an NIH3T3 cell clone transfected with BL-pSV35 and BL-pSV40 (11, 16) was also propagated and measured for comparable expression. As a negative control, a retroviral vector carrying only *Neo* (G1Na, generously provided by G.T.I., Germantown, MD) was used.

IL-12 and IFN-γ assays

To determine the bioactivity of IL-12 produced by transfectants, the proliferative response of day 4 human PHA-activated lymphoblasts was measured as described previously (11). IFN-γ serum measurements were performed using an ELISA kit purchased from Genzyme (Cambridge, MA) with a sensitivity limit of 50 pg/ml. IL-12 levels were expressed as nanograms/10^6 cells/48 h.

Tumor models

The MCA207 methylcholanthrene-induced sarcoma (20) was generously provided by Dr. S. A. Rosenberg (National Cancer Institute, Bethesda, MD) and used throughout the present study. The other cells lines also used in this study were the B16-F10 murine melanoma cell line (generously provided by E. Gorelik, University of Pittsburgh, Pittsburgh, PA), the MCA102 methylcholanthrene-induced sarcoma (21) (also provided by Dr. S. A. Rosenberg), and the TS/A murine breast cancer cell line (generously provided by Dr. Guido Forni, Turin, Italy). Tumor cells were maintained as adherent cultures for early one to three passages after

Table I. *IL-12 secretion by transfectants*

Cells	Vectors	Methods[a]	Selection[b]	Amount (ng)[c]	No. of Experiments	Antitumor Effects In Vivo[d]
3T3	BLpSV35, BLpSV40, and JC125*Neo*	Tr	(+)clone	2.9 ± 1.4	5	(+)
3T3	DFG-mIL-12	I	(−)	8.9 ± 3.4	3	NT
3T3	TFG-mIL-12-*Neo*	I	(+)bulk	104.3 ± 38.9	5	(+)
TIB81	TFG-mIL-12-*Neo*	I	(+)bulk	50.0 ± 25.5	5	(+)
MCA207	TFG-mIL-12-*Neo*	I	(+)bulk	57.0 ± 9.9	4	+
MCA102	TFG-mIL-12-*Neo*	I	(+)bulk	104.5 ± 85.6	3	+
TS/A	TFG-mIL-12-*Neo*	I	(+)bulk	12.8 ± 7.4	2	+
B16-F10	TFG-mIL-12-*Neo*	I	(+)bulk	12.0 ± 2.8	2	+

[a] Tr, transfection using calcium phosphate; I, retroviral infection.

[b] (+), Selected with G418; (−), not selected; clone, a clone was grown from G418-resistant cell populations; bulk, G418-resistant cells without an attempt of cell cloning.

[c] Level of the IL-12 secretion was determined in the supernatant of the cell cultures expressed as the amount of IL-12 per 10^6 cells per 48 h (for details, see *Materials and Methods*). Mean ± SD in the experiments of which numbers are specified in the next column. 0.25 ng = 1 Genetics Institute unit = 30 Hoffmann-La Roche units.

[d] Positive in vivo antitumor effects were observed in the tumor cell types marked as +. Delivery of IL-12 by transfected fibroblasts (NIH3T3 and TIB81, both purchased from ATCC) was observed for antitumor effects by admixing with various tumor cell types marked as (+); NT, not tested.

harvesting fresh tumor maintained by serial transplantation in mice. The cell lines were maintained in culture in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 5.5×10^{-5} M of 2-ME (all obtained from Life Technologies, Inc., Grand Island, NY). Female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and used in experiments at the age of 8 to 12 wk. Groups of four to seven animals were ear tagged and randomized before i.d. inoculation in the shaved area of either flank with tumor cells. The numbers of the animals used in each experiment are specified in the corresponding part of *Results*. The presence of palpable tumor and the size of individual tumors were examined and recorded in a blinded fashion. Each animal experiment was performed at least twice, and the results of a representative experiment are presented. The specific numbers of the attempts performed for individual experiments and inter-experiment differences are described in detail in the appropriate parts of *Results*.

Tumor establishment and vaccination

These experiments were performed to examine the tumorigenicity of cells after gene transfection. A total of 3×10^5 or 5×10^5 live MCA207 tumor cells/animal was injected i.d. and animals were examined for the emergence of palpable tumors. When animals challenged with tumor did not develop a palpable tumor for the first 30 days, then a larger number of nontransfected tumor cells (5×10^5 to 2×10^6) was injected i.d. within the other flank to evaluate the induction of protective immunity against tumor. Similar experiments were performed using the poorly or nonimmunogenic tumor MCA102.

Treatment of tumor at a distant site

On day 0, nontransfected MCA207 tumor cells were inoculated i.d. in the left flank. On day 0 or 3, MCA207 cells with or without IL-12 transfection or saline were injected into the right flank to determine the antitumor effects on the nontransfected cells at a distant site. The serum of treated animals was obtained in some experiments by tail vein phlebotomy.

Blocking Abs

Anti-CD4 (American Type Culture Collection (ATCC), Bethesda, MD) clone GK 1.5, rat IgG2b, and anti-CD8 (ATCC clone 2.43, rat IgG2b) mAb were prepared as previously described (9). These Abs were administered i.p. (1.0 mg) 18 h before cytokine treatment. Two-color flow cytometric analysis was performed using a FACScan (Becton Dickinson, Mountain View, CA) verifying 95% depletion of specific cell subsets in the spleen after the administration of depleting Abs (data not shown). To eliminate NK activity in mice, 20 μl of antiasialo GM1 antiserum (ASGM1, Wako BioProducts, Richmond, VA) were administered i.p. 5 days and 1 day before tumor cell injection, and once every 5 days afterward for an additional 20 days (six times in total). This dose was confirmed to be effective in deleting more than 95% of the lytic activity against YAC-1 cells mediated by spleen cells harvested from the animals receiving a two injection regimen. Murine mAb to IFN-γ and TNF-α were prepared by R. D. Schreiber (see Ref. 22). Mice were injected i.p. with 1.0 ml of purified Ab (0.25 mg/ml) 18 h before the injection of tumor cells. Abs at the same dose were also injected on days 6 and 13. Administration of the same batch of Abs was previously demonstrated in other experiments to be associated with undetectable serum levels of each specific cytokine monitored by ELISA, even under conditions known to induce these cytokines, such as administration of IL-2 or IL-12 (10).

Statistical analysis

Duration before tumor emergence after tumor injection and the size of the tumor at specific dates in individual groups were statistically analyzed using nonparametric tests (Mann-Whitney U test) in representative experiments. The percentage of mice without tumor in each group at certain dates was calculated and compared with other groups using a χ^2 test. In this χ^2 analysis, all the animals in the replicated experiments were included specifying the individual numbers in each single experiment in the text. The differences were considered statistically significant when the p value was less than 0.05.

Results

High level expression of rIL-12 in transfected cells

The level of IL-12 secretion by various transfected cell lines, including NIH3T3, TIB81, MCA207, MCA102, TS/A, and B16F10 cells is shown in Table I. Compared with the expression of the NIH3T3 cells used in the previous studies (11), NIH3T3 cells infected with DFG-mIL-12 expressed twice the level of secreted IL-12 after retroviral infection. When cells were infected with TFG-mIL-12-*Neo* and selected with G418, the level of IL-12 expression was further improved to 104.3 ± 38.9 ng/10^6 cells/48 h. A similar retroviral construct carrying cDNAs of p40, IRES, *Neo*, IRES, and p35 in this order (TFG-*Neo*-mIL-12) was also constructed. Preliminary results showed that the titer and IL-12 expression of cells infected with TFG-*Neo*-mIL-12 were comparable with those of the cells infected with TFG-mIL-12-*Neo* (data not shown). This supports the notion that splicing

events between the IRES domains did not occur. IL-12 expression was also tested for the MCA207, MCA102, B16, and TS/A cell line infected with TFG-mIL-12-*Neo*. All expressed significant amounts of secreted bioactive IL-12. The infected and selected tumors (MCA207-IL-12 and MCA102-IL-12) were used for subsequent experiments in murine tumor models.

Retrovirus-mediated transduction of mIL-12 abrogates tumor establishment

To determine whether retroviral transduction of IL-12 can alter tumor establishment, 3×10^5 nontransduced MCA207, transduced only with *Neo* using G1Na (MCA207-*Neo*), or IL-12 (MCA207-IL-12) were inoculated i.d. in the right flank of C57BL/6 mice (Fig. 2*A*, five animals/group). The growth rates of MCA207, MCA207-*Neo*, and MCA207-IL-12 were not significantly different in culture (data not shown). All of the animals that received nontransduced MCA207 or MCA207-*Neo* developed a palpable tumor by day 10 and these tumors grew progressively. No palpable tumor was observed in any of the animals that were injected with MCA207-IL-12 (through day 33). When an increased number of MCA207-IL-12 cells (5×10^6 cells) were used for injection in similar experiments, some of the animals developed barely palpable tumors (measured up to 5×5 mm) for 7 to 10 days after the tumor inoculation, which all subsequently rejected completely. Those animals that rejected MCA207-IL-12 were rechallenged i.d. in the opposite flank using 5×10^5 nontransduced MCA207, and all animals were immune, rejecting the injected tumor. This experiment was repeated in the same manner in 10 subsequent experiments using five to seven mice in each group. In total, all of the animals that received nontransduced MCA207 (59; 5, 7, 6, 5, 6, 5, 5, 5, 5, 5, 5 in each experiment) or MCA207-*Neo* (58; 5, 7, 5, 5, 6, 5, 5, 5, 5, 5, 5 in each experiment) developed a palpable tumor by day 14 and these tumors grew progressively (59/59 and 58/58, respectively). None of the animals that were injected with MCA207-IL-12 (58; 5, 7, 5, 5, 6, 5, 5, 5, 5, 5, 5 in each experiment) developed progressive tumors, and were tumor-free at the dates later than day 33 (0/58, $p < 0.0001$ for either MCA207 or MCA207-*Neo*). Some of these tumor-free animals were challenged with an unrelated tumor cell line, 1×10^5 of B16-F10, which grew progressively without any discernible delay (10/10). A similar series of experiments was performed using the MCA102 tumor (Fig. 2*B*). A total of 1×10^5 nontransduced MCA102, transduced only with *Neo* (MCA102-*Neo*), or IL-12 (MCA102-IL-12) were inoculated i.d. in the right flank. The growth rates of MCA102, MCA102-*Neo*, and MCA102-IL-12 were not significantly different in culture (data not shown). All of the animals that received MCA102 or MCA102-*Neo* had palpable tumor at day 9 and these tumors grew progressively. In all the animals that were injected with MCA102-IL-12, no palpable tumor was ever observed through day 35. Those animals that rejected MCA102-IL-12 were rechallenged i.d. in the opposite flank using 2×10^5 nontransfected MCA102; four out of five animals (80%) were immune and rejected the rechallenge. In repeated experiments, similar results were obtained (MCA102, 4/4; MCA102-*Neo*, 5/5; MCA102-IL-12, 1/5). In total, the rate of tumor emergence in the animals that received MCA102-IL-12 (1/10) was significantly smaller when compared with

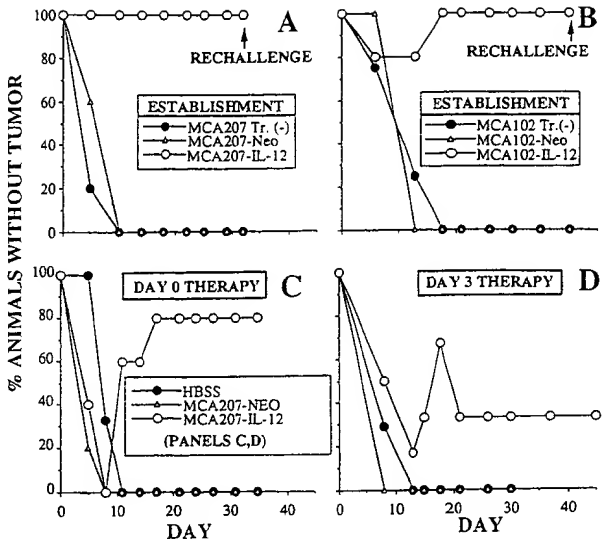

FIGURE 2. Transfection of IL-12 abrogates tumorigenicity of MCA207 and MCA102, and promotes protective and therapeutic antitumor immunity. *A*, IL-12 transfection inhibits tumor establishment of MCA207. C57BL/6 mice were inoculated in the right flank i.d. with 3×10^5 207 tumor cells, which were either not transfected (MCA207), transfected with GTI-*Neo* (MCA207-*Neo*), or transfected with TFG-mIL-12-*Neo* (MCA207-IL-12). Only animals that received MCA207-IL-12 were tumor free at day 32. These animals subsequently received and rejected an inoculation in the left flank (i.d.) with 5×10^5 nontransfected MCA207 tumor cells. Each group consisted of five animals. *B*, IL-12 transfection inhibits tumor establishment of MCA102. The C57BL/6 mice were inoculated in the right flank i.d. with 1×10^5 102 tumor cells, which were either not transfected (MCA102 Tr.-), transfected with GTI-*Neo* (MCA102-*Neo*), or TFG-mIL-12-*Neo* (MCA102-IL-12). All animals that received MCA102-IL-12 were tumor free at day 37. These animals subsequently received i.d. inoculation in the left flank with 2×10^5 nontransfected MCA102 tumor cells, and 80% of the animals (4/5) rejected this subsequent challenge. Each group consisted of four or five animals. *C*, the inoculation of tumor cells transfected with IL-12 affects the growth of the nontransfected tumor at a distant site. C57BL/6 mice were inoculated in the left flank i.d. with 3×10^5 nontransfected 207 tumor cells on day 0. At the same time (day 0), the same animals received HBSS or 3×10^5 207 tumor cells transfected with G1Na or TFG-mIL-12-*Neo* in the right flank i.d. The percent of animals without palpable tumor in the left side is depicted. *D*, inoculation of tumor cells transfected with IL-12 has a therapeutic effect on the growth of the established day 3 nontransfected tumor inoculated i.d. in the opposite flank.

MCA102 (9/9, $p = 0.0001$) or MCA102-*Neo* (10/10, $p = 0.0001$).

Inoculation of MCA207-IL-12 suppresses the growth of nontransfected MCA207 at a distant site

MCA207 tumor cells (3×10^5) were inoculated i.d. in the left flank of C57BL/6 mice on day 0. At the same time on day 0, HBSS (6 animals) or 3×10^5 MCA207-*Neo* (5 animals), MCA207-IL-12 (5 animals) cells were inoculated in the right flank of the same animals (Fig. 2*C*). Although the inoculated MCA207-*Neo* grew progressively, MCA207-IL-12 failed to grow (data not shown). The nontransfected MCA207 inoculated in the left flank of animals treated with HBSS or MCA207-*Neo* grew progressively, but 4 out of 5 animals treated with MCA207-IL-12 rejected the nontransfected tumor when injected in the opposite side. In a subsequent experiment, 3 of 5 animals that received MCA207-IL-12 in the right flank rejected nontransfected MCA207 inoculated in the left flank, whereas none of the animals treated with HBSS (5 animals) or MCA207-*Neo* (5 animals) rejected tumor in the left flank. In total, 7 of 10 animals treated with MCA207-IL-12 rejected nontransfected MCA207 at the distant site, whereas none of the animals treated with either HBSS (0/11) or MCA207-*Neo* (0/10) did ($p = 0.007$ and $p = 0.01$, respectively).

In a similar model, 3×10^5 nontransfected MCA207 were injected into the left flank at day 0, and HBSS (7 animals), 5×10^6 MCA207-*Neo* (7 animals) or MCA207-IL-12 (6 animals) cells were injected i.d. in the right flank at day 3 (Fig. 2*D*). Only the animals treated with MCA207-IL-12 rejected the established nontransfected tumor (2 of 6), and the size of the tumor at day 21 (mean = 15.2 ± 13.4 mm^2) was significantly smaller than that observed in animals treated with HBSS (mean = 46.3 ± 31.7 mm^2; $p = 0.038$) or with MCA207-*Neo* (mean = 51.0 ± 18.6 mm^2; $p = 0.0066$). In another experiment, similar effects were observed in the animals treated with MCA207-IL-12, but there was no effect upon tumor growth at a distant site in the animals that received injection with an identical number of irradiated nontransduced MCA207 cells as a control (data not shown). Cumulatively in a total of three experiments conducted in day 3 tumor models, including this representative experiment, similar effects were observed on nontransduced tumor at a distant site (animals free of tumor/total animals in the group = 2/6, 2/5, and 1/5, respectively). In total, the percentage of the animals that rejected day 3 nontransfected tumor at the distant site was significantly higher when treated with MCA207-IL-12 when compared with those treated with HBSS ($p < 0.001$) or MCA207-*Neo* ($p < 0.001$). At day 4 of the experiments shown in Figure 2*C*, serum samples were collected and measured for IFN-γ. None of the serum samples from any animals in this group displayed detectable levels of IFN-γ (<50 pg/ml, data not shown).

CD4$^+$ and CD8$^+$ cell depletion or NK depletion alone only partially abrogate the antitumor effect of IL-12 transfection

To examine the mechanism by which IL-12 mediates its antitumor effects on tumor establishment, CD4$^+$ and CD8$^+$ T cells were depleted in vivo using specific Abs administered i.p. (Fig. 3*A*). Only these animals that received MCA207-*Neo* (i.d.) and HBSS (i.p.), or MCA207-IL-12 (i.d.) and both anti-CD4$^+$ and CD8$^+$ Abs (i.p.), developed tumors. Although all of the animals in the latter group eventually developed palpable tumors, the time of emergence of palpable tumor (mean ± SD = 29.8 ± 7.9 days) was significantly delayed ($p = 0.009$) and the size of the tumors at day 32 (mean = 17 ± 13.9 mm^2) was significantly smaller ($p = 0.009$) when compared with those of animals receiving MCA207-*Neo* (i.d.) and HBSS (i.p.) (mean = 9 ± 0 days and 94.4 ± 42.8 mm^2) suggesting the role of additional antitumor effector cells. To examine the role of the NK cells on tumor establishment, anti-ASGM1 serum was administered with or without Abs to CD4$^+$ and CD8$^+$ (Fig. 3*B*). When animals received MCA207-IL-12 (i.d.) and Ab to ASGM1 alone, a somewhat larger percentage (80%) of animals displayed palpable tumors and tumors were also larger (mean = 10 ± 6.6 mm^2) at day 9 ($p = 0.144$) compared with animals receiving MCA207-IL-12 (i.d.) and HBSS (i.p.) (40% and 3.6 ± 4.9 mm^2). However, these differences are not statistically significant, and the tumors in all animals in these two groups eventually disappeared. Again, in the animals that received MCA207-IL-12 (i.d.) and both anti-CD4$^+$ and CD8$^+$ Abs (i.p.), the emergence of palpable tumors (mean = 14.2 ± 7.4 days) was delayed ($p = 0.060$) and the size of tumors on day 19 (mean = 22.2 ± 15.1 mm^2) was smaller ($p = 0.012$) compared with those in animals that received MCA207-*Neo* (i.d.) and HBSS (i.p.) (mean = 6.6 ± 1.3 days and 62.6 ± 22.6 mm^2). Surprisingly, tumors in animals receiving MCA207-IL-12 (i.d.) and all three Abs (against CD4$^+$, CD8$^+$, and ASGM1) (i.p.) grew progressively (7.2 ± 1.6 days for detection and with a mean size of 79.2 ± 13.9 mm^2) with kinetics comparable with those observed for animals injected with MCA207-*Neo* and treated with HBSS ($p = 0.602$ and $p = 0.251$, respectively).

Neutralization of IFN-γ partially abrogates the antitumor effect of IL-12 transfection whereas neutralization of TNF-α does not

To examine the role of IFN-γ and/or TNF-α in the antitumor effects of IL-12 transduction observed in tumor establishment, cytokine-specific neutralizing Abs with the schedule described in *Materials and Methods* (Fig. 3*C*) were administered. All of the animals in the group receiving MCA207-IL-12 (i.d.) and Ab to IFN-γ yielded palpable tumors at day 9 after tumor inoculation, whereas only one (20%) of those in the MCA207-IL-12 (i.d.) group treated with HBSS developed a palpable tumor. The mean

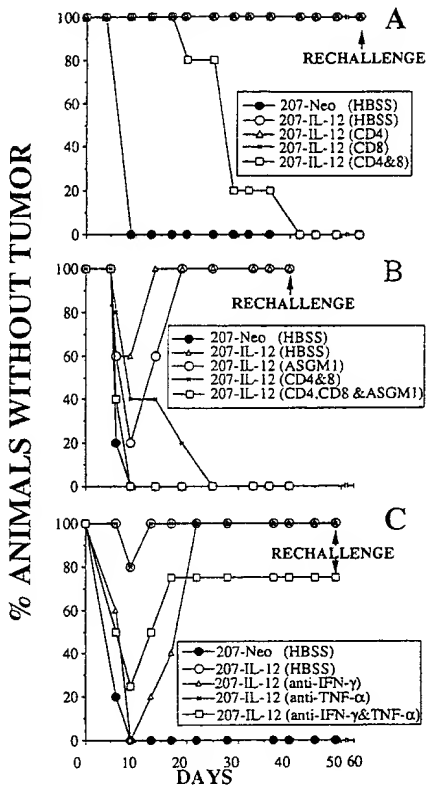

FIGURE 3. The antitumor effects of IL-12 transfection are mediated by T cells and NK cells partially requiring IFN-γ. *A*, depletion of both $CD4^+$ and $CD8^+$ cells partially abrogates the antitumor effects of IL-12 paracrine secretion. The animals free of palpable tumors subsequently received and rejected an inoculation in the left flank (i.d.) with 1×10^6 nontransfected MCA207 tumor cells. Each group consisted of five animals. *B*, depletion of $CD4^+$, $CD8^+$, and NK cells (ASGM1) can completely abrogate the antitumor effects induced by IL-12 gene transfection. The animals free of palpable tumors subsequently received and rejected an inoculation in the left flank (i.d.) of 1×10^6 non-transfected MCA207 tumor cells. Each group consisted of five animals. *C*, delivery of IFN-γ neutralizing Abs partially abrogates the antitumor effects induced by IL-12, particularly during the early phase of the immune rejection. The animals free of palpable tumors also subsequently received and rejected an inoculation in the left flank (i.d.) of 1×10^6 nontransfected MCA207 tumor cells. Each group consisted of five animals.

tumor area on day 9 in the anti-IFN-γ-tested group was 14.6 ± 5.2 mm^2, which was similar to the tumor area in those animals that received MCA207-*Neo* cells (i.d.) and HBSS (i.p.) injections (mean = 20 ± 11.3 mm^2), but was significantly larger ($p = 0.009$) than the tumor area of those animals that received MCA207-IL-12 cells (i.d.) and HBSS injection (i.p.) (0.8 ± 1.8 mm^2). In the anti-TNF-α Ab-treated animals, the percentage of animals with palpable tumors (20%) and the mean tumor area (1.2 ± 2.7 mm^2) were similar to those in the group receiving MCA207-IL-12 cells (i.d.) and HBSS injection ($p = 0.917$). When both anti-IFN-γ and anti-TNF-α Abs were administered, 75% of the animals receiving MCA207-IL-12 cells and HBSS had palpable tumors at day 9 and the tumor area at that time was 13.3 ± 13.1 mm^2, comparable with that of the anti-IFN-γ alone-treated group ($p = 1.00$). Most of the animals that received anti-IFN-γ Abs, with or without the anti-TNF-α Ab, eventually rejected tumor. Only one animal in this group suffered from progressively growing tumor and was killed on day 37 after tumor injection. The animals free of palpable tumors in this experiment rejected a rechallenge with 1×10^6 nontransfected MCA207 cells in the left flank. Repeated experiments using five animals/group yielded similar results. In brief, all of the animals that received MCA207-IL-12 and anti-IFN-γ Ab alone or both anti-IFN-γ and anti-TNF-α Abs had palpable tumors for less than 14 days, and eventually rejected tumor (5/5 and 5/5, respectively).

Discussion

An understanding of the mechanisms by which some animals reject tumor whereas others display progressive tumor outgrowth is gradually evolving based on an appreciation of the underlying precepts of cellular and tumor immunology. Molecular analysis of the cytokines elaborated, evaluation of infiltrating lymphoid subsets, and studies of Ag processing and presentation have all been informative in various tumor types in mice and humans. Although IFN-γ has been shown to be critical for tumor rejection, allograft rejection, and some autoimmune phenomena, its systemic administration has paradoxically failed to limit tumor growth (23–25). Because IL-12 is the single most potent inducer of IFN-γ production by T and NK cells, an examination of its local effects on antitumor immunity assessed using retroviral transfection is of significant scientific interest and potentially of clinical interest.

We have constructed a retroviral vector that results in high level secretion of bioactive mIL-12 at high levels after infection. The simultaneous expression of the p35 and p40 subunits was achieved using two IRES sequences from EMCV giving rise to a polycistronic, LTR-based transcript encoding p40, p35, and *Neo*. This was done in part to avoid the potential mutual interference among multiple promoters (15). One potential problem with this TFG vector system could be homologous recombination of the two identical IRES sequences resulting in the deletion of the second (i.e., p35) gene product. Because the titer of the TFG vector was comparable with that of other DFG vectors that have only one IRES sequence and because we were able to obtain large amount of bioactive IL-12 from the target cells after *Neo* selection, this potential problem did not appear to adversely affect our model system. An additional concern arose about the stoichiometry of the p40 and p35 subunits expressed by the TFG-mIL-12-*Neo* vector. An excess of p40 expression compared with that of p35 could represent a significant problem because excess p40, probably active as a homodimer, has recently been

shown to inhibit IL-12 heterodimer in murine studies (26, 27). Because p40 was located in the first subcloning position of the TFG-mIL-12-*Neo* construct, it was anticipated that the translation of the p40 gene product might exceed that of the p35 gene product (14, 15, 26, 28). In each experiment, the secretion of mIL-12 by transfected cells was confirmed in a PHA blast bioassay. Although an excess of p40 expression could be a potential problem in murine TFG-mIL-12 transfection studies, the inhibitory effect of free p40 has not been observed in humans (26, 28) (L. Zitvogel, unpublished observation) (G. Trinchieri and S. F. Wolf, personal communication). Naturally produced hIL-12, which is bioactive, contains free p40 (2). Thus a retroviral vector containing hIL-12 cDNAs (TFG-hIL-12-*Neo*) has been constructed for a proposed human clinical trial, and has been characterized in detail (14).

Using the TFG-mIL-12-*Neo* retroviral system, we have shown here that MCA207 and MCA102 tumors transfected with IL-12 fail to establish palpable tumor in syngeneic animals and that these resulting animals can reject a subsequent challenge with the corresponding nontransfected tumor cells. Many maneuvers, including the use of *Corynebacterium parvum* as a potent adjuvant with irradiated cells, have failed to generate such protection to MCA102 (21). Thus, IL-12 paracrine secretion appears to be very potent in induction of systemic immunity even for poorly immunogenic tumor. A similar effect has been observed in analogous experiments using other tumors (TS/A, B16F10; data not shown). The inoculation of IL-12-transfected live cells also caused regression of a nontransfected tumor inoculated either at the same time as, or up to 3 days before, the therapeutic tumor inoculum. Therapeutic antitumor effects were observed in the day 0 therapy model when relatively low numbers of tumor cells (3×10^5 cells/animal) were administered. This number of cells can secrete about 17 ng IL-12/48 h in vitro, which is approximately 10-fold less than that amount determined to exert minimal antitumor effects when administered systemically as reported in our previous studies (10). Thus, the antitumor effects appear to be mediated primarily by a specific immune response of the host. However, it is possible that some of the antitumor effects in the current model might be caused by the antitumor effects of IL-12 produced and released into the blood stream from the transfected cells, particularly when the larger number of MCA207-IL-12 cells were used in the inoculum. The serum concentration of IFN-γ, which can be induced by systemic administration of IL-12 protein at high doses in vivo (8, 10), remained below the detectable levels at 4 days after implantation of 5×10^6 MCA207-IL-12 cells. This suggests that the IL-12 concentration in the serum was not high enough to induce systemic production of IFN-γ. To address this issue more precisely, we are planning to measure directly IL-12 concentration in the serum of the animals with gene therapy at various time points and compare with that of the animals with systemic IL-12 protein administration. These studies should also enable us to compare directly the therapeutic efficacy between IL-12 gene therapy and systemic administration of IL-12 protein. Preliminary study showed that IL-12 was detectable at very limited time points in the serum of the animals injected with relatively large number of the cells (3×10^6 cells of IL-12 transduced cells/animal).

The immune mechanisms responsible for the antitumor effects of IL-12 have been previously examined in murine models using systemic administration of IL-12. Brunda et al. (9) showed that the IL-12 antitumor effect was impaired in T cell-deficient nude mice, but not in beige mice, which are relatively NK deficient. From these results, and from studies using NK-depleting Abs, it was suggested that T cells, but not NK cells, were essential for the antitumor effects of IL-12 given systemically (9). We have also shown that the effects of systemically administered IL-12 can be abrogated using sublethal total body radiation, and partially inhibited by administration of neutralizing Abs to IFN-γ. $CD4^+$ and $CD8^+$ T cell depletion also effectively abrogated the IL-12-induced antitumor effects (10). The immune mechanism responsible for the antitumor effects observed with systemic rIL-12 have not been examined in murine therapeutic models using paracrine IL-12 delivery. When both $CD4^+$ and $CD8^+$ T cells were depleted, transfected tumor cells were ultimately able to grow, but the time of emergence of palpable tumor after inoculation was significantly delayed and the size of the injected MCA207-IL-12 tumor was significantly smaller when compared with the nontransfected control. The IL-12-induced antitumor effects were completely abrogated only when NK cells and each of the two predominant T cell populations were simultaneously eliminated. These results suggest that NK cells play a role in the antitumor effects of IL-12, principally during the early phase of antitumor reactivity. T cells play a major role in the latter stage resulting in the final eradication of tumor and acquisition of host immunity.

Animals that were depleted of either $CD4^+$ or $CD8^+$ cells alone successfully rejected a subsequent challenge with nontransfected tumor cells. These results suggest that either $CD4^+$ or $CD8^+$ cells alone, along with IL-12, were sufficient not only for abrogating tumor establishment but also for inducing protective antitumor immunity. This finding is quite different from that observed for other cytokines such as granulocyte-macrophage-CSF (17) in which effects can be blocked by the depletion of either one of these T cell subsets. Furthermore, IFN-γ neutralization partially suppresses the effectiveness of IL-12 in tumor establishment models but fails to abrogate its effects on promoting protective immunity. These results suggest that IFN-γ plays a critical role during the early phase of tumor growth, but not necessarily in the latter phase. Such findings correlate well with the findings in immunization models using a hapten-protein conjugate (29); effects of IL-12

including the induction of Th1 development is not prevented by co-administration of neutralizing Abs to IFN-γ (29). However, it is possible for most of the in vivo abrogation of the cytokine with Ab administration that the abrogation of IFN-γ might not be complete throughout the process. To avoid this problem, further studies using the animals with targeted disruption of IFN-γ production is appropriate for confirmation of our current observation.

The findings described in this study have extended and clarified the results of our earlier studies using paracrine or systemic administration of IL-12 (10, 11) and allows us the opportunity to speculate on the mechanisms of IL-12-induced antitumor immunity. At the time of the inoculation of tumor cells transfected with IL-12, locally secreted IL-12 initially activates NK cells. These activated NK cells secrete soluble factors including IFN-γ, which are likely involved in the observed tumor-suppressive effects induced by IL-12. However, these early events do not appear to critically affect the induction of specific immunity mediated by tumor-specific $CD4^+$ and/or $CD8^+$ T cells. Antitumor T cells can be generated in vivo in the absence of discernible NK cells or IFN-γ. The ability of IL-12 to promote predominantly a Th1-type response (7) cannot explain the observed antitumor effects completely, because neutralization of IFN-γ or depletion of either $CD4^+$ or $CD8^+$ T cells alone failed to abrogate the IL-12-induced antitumor effects. It is likely that, in addition to IFN-γ, IL-12 might induce other currently known or unknown cytokines from $CD4^+$ T cells, $CD8^+$ T cells, and/or NK cells that are critically involved in the development of an effective antitumor immune response. To address this possibility, we are currently examining the expression level of known cytokines induced by IL-12 using quantitative PCR techniques and are evaluating novel (cytokine) gene products stimulated by IL-12 in both T cells and NK cells. p40 knockout mice, recently created by targeted disruption will be helpful in resolving what role IL-12 plays in the natural development of an immune response. Because we have now confirmed and extended the findings that mIL-12 paracrine administration mediates potent antitumor effects in vivo in diverse tumor cell types evaluated in multiple murine strains, we are hopeful to translate the present study into effective clinical treatments.

Acknowledgments

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References

1. Kobayashi, M., L. Fitz, M. Ryan, R. M. Hewick, S. C. Clark, S. Chang, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med. 170:827.*
2. Stern, A. S., F. J. Podlaski, J. D. Hulmes, Y. C. E. Pan, P. M. Quinn, A. G. Wolitzky, P. C. Familletti, D. L. Stremlo, T. Truitt, R. Chizzonite, and M. K. Gately. 1990. Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. *Proc. Natl. Acad. Sci. USA 87:6808.*
3. Wong, H. L., D. E. Wilson, J. C. Jenson, P. C. Familletti, D. L. Stremlo, and M. K. Gately. 1988. Characterization of a factor(s) which synergizes with recombinant interleukin 2 in promoting allogeneic human cytolytic T-lymphocyte responses in vitro. *Cell Immunol. 111:39.*
4. Chan, S. H., B. Perussia, J. W. Gupta, M. Kobayashi, M. Pospisil, H. A. Young, S. F. Wolf, D. Young, S. C. Clark, and G. Trinchieri. 1991. Induction of interferon-γ production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. *J. Exp. Med. 173:869.*
5. Naume, B., M. Gately, and T. Espevik. 1992. A comparative study of IL-12 (cytotoxic lymphocyte maturation factor), IL-12 and IL-7 induced effects on immunomagnetically purified $CD56^+$ NK cells. *J. Immunol. 148:2429.*
6. Gately, M. K., B. B. Desai, A. G. Wolitzky, P. M. Quinn, C. M. Dwyer, F. J. Podlaski, P. C. Familletti, F. Sinigalia, R. Chizzonite, U. Gubler, and A. S. Stern. 1991. Regulation of human lymphocyte proliferation by a heterodimeric cytokine, IL-12 (cytotoxic lymphocyte maturation factor). *J. Immunol. 147:874.*
7. Hsieh, C-S., S. Macatonia, C. Tripp, S. Wolf, A. O'Garra. and K. Murphy. 1993. Development of Th1 $CD4^+$ T cells through IL-12 produced by listeria-induced macrophages. *Science 260:547.*
8. Gately, M. K., R. R. Warrier, S. Honasoge, D. A. Faherty, S. E. Connaughton, T. D. Anderson, U. Sarmiento, B. R. Hubbard, and M. Murphy. 1994. Administration of recombinant IL-12 to normal mice enhances cytolytic lymphocyte activity and induces production of IFN-γ in vivo. *Int. Immunol. 6:157.*
9. Brunda, M. J., L. Luistro, R. R. Warrier, R. B. Wright, B. R. Hubbard, M. Murphy, S. F. Wolf, and M. K. Gately. 1993. Antitumor and antimetastatic activity of interleukin-12 against murine tumors. *J. Ex. Med. 178:1223.*
10. Nastala, C. L., H. Edington, W. Storkus, T. G. McKinney, H. Tahara, M. J. Brunda, M. K. Gately, R. Schreiber, and M. T. Lotze. 1994. Recombinant interleukin-12 administration induces tumor regression in association with interferon-gamma and nitric oxide production. *J. Immunol. 153:1697.*
11. Tahara, H., H. J. Zeh III, W. J. Stokus, I. Pappo, S. C. Watkins, U. Gubler, S. F. Wolf, P. D. Robbins, and M. T. Lotze. 1994. Fibroblasts genetically engineered to secrete interleukin-12 can suppress tumor growth and induce anti-tumor immunity to a murine melanoma in vivo. *Cancer Res. 54:182.*
12. Wolf, S. F., P. A. Temple, M. Kobayashi, E. Young, M. Dicig, L. Lowe, R. Dzialo, L. Fitz, C. Ferenz, R. M. Hewick, K. Kelleher, S. H. Herrmann, S. C. Clark, L. Azzoni, S. H. Chan, G. Trinchieri, and B. Perussia. 1991. Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effect on T and natural killer cells. *J. Immunol. 146:3074.*
13. Gubler, U., A. O. Chua, D. S. Schoenhout, C. M. Dwyer, W. McComas, R. Motyka, N. Nabavi, A. G. Wolitzky, P. M. Quinn, P. C. Familletti, and M. K. Gately. 1991. Coexpression of two distinct genes is required to generate secreted bioactive cytotoxic lymphocyte maturation factor. *Proc. Natl. Acad. Sci. USA 88:4143.*
14. Zitvogel, L., H. Tahara, G. Mueller, S. F. Wolf, U. Gubler, M. K. Gately, P. D. Robbins, and M. T. Lotze. 1994. Construction and characterization of retroviral vectors expressing biologically active human IL-12. *Human Gene Therapy 5:1493.*
15. Ghattas, I. R., J. R. Sanes, and J. E. Majors. 1991. The encephalomyocarditis virus internal ribosome entry site allows efficient coexpression of two genes from a recombinant provirus in cultured cells and in embryos. *Mol. Cell. Biol. 11:5848.*

16. Morgan, R. A., L. Couture, O. Eloroy-Stein, J. Ragheb, B. Moss, and W. F. Anderson. 1992. Retroviral vectors containing putative internal ribosome entry sites: development of a polycistronic gene transfer system and applications to human gene therapy. *Nucleatic Acids Res. 20:1293.*
17. Dranoff, G., E. Jaffee, A. Lazenby, P. Golumbek, H. Levitsky, K. Brose, V. Jackson, H. Hamada, D. Pardoll, and R. Mulligan. 1993. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. USA 90:3539.*
18. Danos, O., and R. C. Mulligan. 1988. Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. *Proc. Natl. Acad. Sci. USA 85:6460.*
19. Pear, W. S., G. P. Nolan, M. L. Scott, and D. Baltimore. 1993. Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. USA 90:8392.*
20. Shu, S., and S. A. Rosenberg. 1985. Adoptive immunotherapy of newly induced murine sarcomas. *Cancer Res. 45:1647.*
21. Mule, J. J., J. C. Yang, R. L. Lafreniere, S. Y. Shu, and S. A. Rosenberg. 1987. Identification of cellular mechanisms operational in vivo during the regression of established pulmonary metastases by the systemic administration of high-dose recombinant interleukin-2. *J. Immunol. 139:285.*
22. Sheehan, K. C. F., N. H. Ruddle, and R. D. Schreiber. 1989. Generation and characterization of hamster monoclonal antibodies that neutralize murine tumor necrosis factors. *J. Immunol. 142:3884.*
23. Weiner, L. M. 1991. Applications of gamma-interferon in cancer therapy. *Mol. Biotherapy 3:186.*
24. Jacob, C. O. 1992. Tumor necrosis factor and interferon gamma: relevance for immune regulation and genetic predisposition to auto immune disease. *Semin. Immunol. 4:147.*
25. O'Connell, P. J., A. Pacheco-Silva, P. W. Nickerson, R. A. Muggia, M. Bostos, V. R. Kelly, and T. B. Strom. 1993. Unmodified pancreatic islet allograft rejection results in the preferential expression of certain T cell activation transcripts. *J. Immunol. 150:1093.*
26. Mattner, F., S. Fischer, S. Guckes, S. Jin, H. Kaulen, E. Schmitt, E. Rude, and T. Germann. 1993. The interleukin-12 subunit p40 specifically inhibits effects of the interleukin-12 heterodimer. *Eur. J. Immunol. 23:2202.*
27. Gillessen, S., D. Carvajal, P. Ling, F. J. Podlaski, D. L. Stremlo, P. C. Familletti, V. Gubler, D. H. Presky, A. S. Stern, and M. K. Gately. 1995. Mouse interleukin-12 (IL-12) p40 homodimer: a potent IL-12 antagonist. *Eur. J. Immunol. 25:200.*
28. Chizzonite, R., T. Truitt, B. B. Desai, P. Nunes, F. J. Podlaski, A. S. Stern, and M. K. Gately. 1992. IL-12 receptor. I. Characterization of the receptor on phytohemagglutinin-activated human lymphoblasts. *J. Immunol. 148:3117.*
29. McKnight, A. J., G. J. Zimmer, I. Fogelman, S. F. Wolf, and A. K. Abbas. 1994. Effects of IL-12 on helper T cell-dependent immune responses in vivo. *J. Immunol. 152:2172.*

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Gene gun-mediated skin transfection with interleukin 12 gene results in regression of established primary and metastatic murine tumors

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ABSTRACT **Particle-mediated (gene gun) *in vivo* delivery of the murine interleukin 12 (IL-12) gene in an expression plasmid was evaluated for antitumor activity. Transfer of IL-12 cDNA into epidermal cells overlying an implanted intradermal tumor resulted in detectable levels (266.0 ± 27.8 pg) of the transgenic protein at the skin tissue treatment site. Despite these low levels of transgenic IL-12, complete regression of established tumors (0.4–0.8 cm in diameter) was achieved in mice bearing Renca, MethA, SA-1, or L5178Y syngeneic tumors. Only one to four treatments with IL-12 cDNA-coated particles, starting on day 7 after tumor cell implantation, were required to achieve complete tumor regression. This antitumor effect was $CD8^+$ T cell-dependent and led to the generation of tumor-specific immunological memory. By using a metastatic P815 tumor model, we further showed that a delivery of IL-12 cDNA into the skin overlying an advanced intradermal tumor, followed by tumor excision and three additional IL-12 gene transfections, could significantly inhibit systemic metastases, resulting in extended survival of test mice. These results suggest that gene gun-mediated *in vivo* delivery of IL-12 cDNA should be further developed for potential clinical testing as an approach for human cancer gene therapy.**

Interleukin 12 (IL-12), a bimolecular glycoprotein consisting of a 35- and a 40-kDa subunit, was originally identified as a factor that stimulates natural killer cells (1, 2) and promotes maturation of cytotoxic T lymphocytes (CTL) (3, 4). It has recently been demonstrated that local or systemic treatment with recombinant (r) IL-12 protein mediates profound antitumor effects *in vivo*, causing regression of established subcutaneous tumors and tumor metastases (5, 6). However, systemic administration of rIL-12 caused dose-dependent toxicity in mice (7) and in human trials (8). Thus, a delivery mechanism that can provide relatively low levels of IL-12 at the target tissue might be advantageous in that it could generate an antitumor effect without causing systemic toxicity. Indeed, as cancer gene therapy has evolved, recent studies have produced encouraging results, showing that murine fibroblasts (9) or tumor cells (10) transduced *in vitro* with the IL-12 gene using a retroviral vector were able to induce antitumor immune responses. These data suggest that peritumoral IL-12 delivery may be as efficacious as systemic administration and avoid many undesirable side effects.

The particle-mediated method for gene delivery by gene gun utilizes a shock wave to accelerate DNA-coated gold particles into target cells or tissues. At submicrogram quantities of DNA per dose for *in vitro* or *in vivo* gene transfer, the gene gun can deliver thousands of DNA copies intracellularly into test tissues, resulting in high level transgene expression (11). As this method is cell surface receptor-independent, it can successfully deliver genes into a wide spectrum of mammalian cell types (12, 13). We have recently demonstrated that a particle-mediated, *in vivo* cytokine gene therapy reduces tumor growth in mice (14). Treatments with interferon-γ and tumor necrosis factor α shortly after the implantation of tumor cells inhibited tumor growth and prolonged the survival of tumor-bearing mice. To more closely approximate clinical situations, and to take advantage of the findings that IL-12 more effectively stimulates activated than naive T cells (15, 16), we evaluated the effect of IL-12 gene therapy on the growth of established tumors. In this study, we utilized the gene gun technology for *in vivo* IL-12 cDNA delivery into the skin overlying the implanted, established tumor tissues.

MATERIALS AND METHODS

Mice. BALB/c, C57BL/6, DBA/2, and A/J female mice between 8 and 12 weeks of age were obtained from Harlan–Sparague–Dawley or Taconic Farms. All animal experiments were conducted in accordance with principles stated in ref. 17.

Murine Tumor Models. Six established mouse tumor cell lines were employed in this study, namely Renca carcinoma, MethA sarcoma (both syngeneic in BALB/c mice), L5178Y lymphoma, P815 mastocytoma (both syngeneic in DBA/2 mice), SA-1 sarcoma, and B16 melanoma (syngeneic in A/J and C57BL/6 mice, respectively). Tumor cell cultures were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine, and gentamicin at 50 μg/ml. MethA sarcoma, SA-1 sarcoma, P815 mastocytoma, and L5178Y lymphoma were grown as ascites in syngeneic mice for 1 week before injecting intradermally (i.d.). Mice were shaved in the abdominal area and injected i.d. with 1×10^6 (or 1×10^5, in the case of B16 tumor) tumor cells in 50 μl phosphate-buffered saline (PBS). Tumor growth was monitored two to three times a week by measuring two perpendicular tumor diameters using calipers.

IL-12 Gene Expression Vector. We constructed a plasmid (pWRG3169) containing coding sequences for the p35 and p40 subunits of murine (m) IL-12, linked tandomly in the same direction and each driven by its own cytomegalovirus (CMV) i/e promoter/enhancer, a simian virus 40 (SV40) sd/sa intron sequence, and a bovine growth hormone polyadenylylation sequence. The murine 35- and 40-kDa IL-12 subunit cDNA clones were isolated from mouse lymphocyte cDNA libraries by PCR cloning. The PUC19 plasmid backbone was derived from a bluescript SK(+) vector with an ampicillin-resistance gene (see Fig. 1*A*). A control vector containing a luciferase

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Abbreviations: IL-12, interleukin 12; CTL, cytotoxic T lymphocyte; mAb, monoclonal antibody; r, recombinant; m, murine; CMV, cytomegalovirus; SV40, simian virus 40; Luc, luciferase.

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(Luc) cDNA expression plasmid containing the CMV promoter was constructed as described by Cheng *et al.* (12).

***In Vivo* and *in Vitro* Gene Transfer.** The experiments utilized a helium-pulse Accell (gene gun) device that was designed by D. McCabe (Agracetus, Inc). Plasmid DNA was precipitated onto 2 μm gold particles. Particles were suspended in a solution of 0.1 mg of polyvinyl pyrrolidone per ml in absolute ethanol. This DNA/gold/particle preparation was coated onto the inner surface of a Tefzel tubing by using a tube loader (Agracetus), and the tubing was cut into 0.5-inch segments to result in delivery of 0.5 mg gold and 1.25 μg plasmid DNA per transfection. For tumor therapy, mouse skin overlying and surrounding the target tumor was transfected *in vivo* with IL-12 or Luc cDNA expression vectors starting from day 7 after i.d. implantation of 1×10^6 of five different types of tumor cells, except for B16 tumor, which was implanted at 10^5 cells. Each treatment consisted of four transfections (5 μg plasmid DNA/treatment) with a 300 psi helium gas pulse. One transfection was directly over the tumor site, and three additional treatments were evenly spaced around the circumference of the tumor in a triangle pattern. The *in vitro* particle bombardment gene transfer was performed as described (18).

IL-12 Bioassay. For determining transgenic IL-12 expression following *in vivo* gene transfer, blood was obtained by cardiac puncture, and skin tissue samples containing four transfection sites were collected in 0.5 ml of general extraction buffer, thoroughly minced with scissors, and sonicated before collecting the supernatant. The level of transgenic IL-12 protein was determined by a cell proliferation bioassay by using murine Con A-activated splenocytes as described (19). Briefly, spleen cells (5×10^6/ml) from naive BALB/c mice were stimulated with Con A (5 μg/ml) for 4 days at 37°C. Serial dilutions of the test samples (cell culture supernatants, serum, or skin tissue homogenates) were incubated with the activated spleen cells (2×10^4 cells/well) for 48 h, and the level of cell proliferation was measured by [^{3}H]thymidine incorporation. Serially diluted recombinant murine IL-12 (R & D Biosystems) was used as a standard. Anti-mIL-12 monoclonal antibody (mAb) (kindly provided by M. Gately, Hoffman–La Roche) was used to ensure that the bioactivity of the samples was due to IL-12. The sensitivity of this assay was about 10 pg/ml for rIL-12 standard protein and cell culture samples, and about 100 pg/ml for serum and skin tissue extracts.

Immunohistochemistry. The *in vivo* transfected skin tissues were sectioned in a cryostat (8 mm), placed on silanated slides and allowed to air dry. Test tissues were then fixed with acetone at 4°C for 10 min, air dried, washed in PBS for 10 min, and incubated with the anti-IL12 mAb (10 mg/ml) for 60 min at room temperature. Reacted tissues were rinsed two times in PBS and incubated with a biotinylated secondary antibody (rabbit anti-rat IgG; Vector Laboratories) for 60 min at room temperature. After rinsing with PBS, localization of the antibody binding was visualized with peroxidase staining and developed with metal enhanced 3,3-diaminobenzidine-H_2O_2. Sections were rinsed in PBS and counterstained with hematoxylin, dehydrated in ethanol, treated with xylene, and mounted with permount.

IL-12 Gene Therapy of Spontaneous Metastasis. A spontaneous metastasis model using weakly immunogenic P815 tumor has been described (20). DBA/2 mice were injected i.d. with 1×10^6 P815 cells. The skin overlying and surrounding the tumor was transfected with IL-12 cDNA or Luc cDNA on days 12 and 14 of tumor growth. Surgical excision of the tumor was performed on day 15 of tumor growth, and additional transfections of the skin on both sides of abdomen were performed on days 16, 18, and 20. Survival of the mice was followed.

Generation of Cytotoxic T Lymphocytes and Cytotoxic Assay. Tumor-specific CTL were generated *in vitro* as described (21). Briefly, spleen cells (5×10^6), derived from BALB/c mice that had rejected Renca tumors due to IL-12 gene therapy and had remained tumor-free for 2 months, or from age-matched naive mice, were cocultured with 5×10^4 mytomicin C-treated Renca cells in 24-well culture plates in complete RPMI 1640 media. After culturing for 5 days *in vitro*,

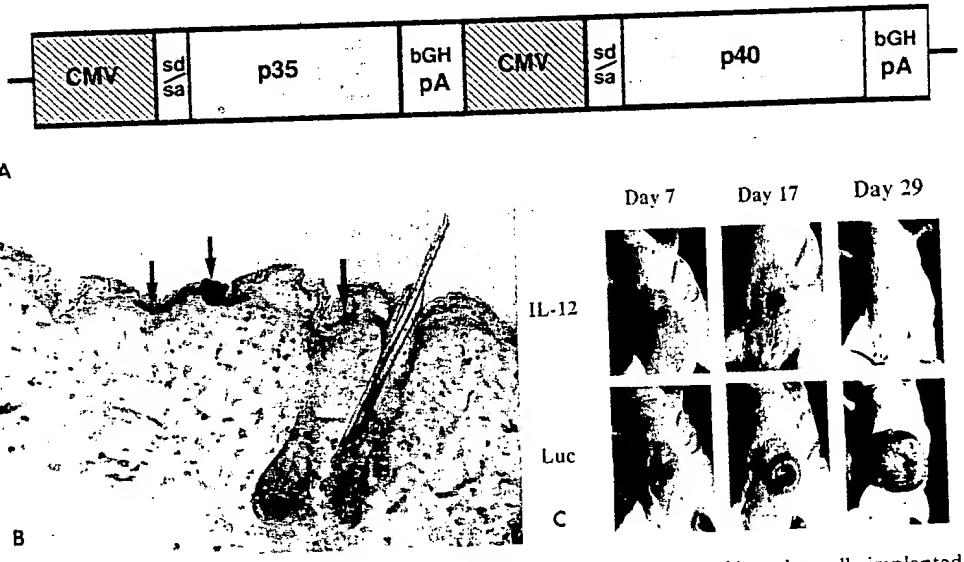

FIG. 1. *In vivo* transfer of IL-12 cDNA expression plasmid into mouse skin leads to regression of intradermally implanted, established tumors. (*A*) IL-12 cDNA gene construct engineered in the pWRG 3169 expression plasmid. CMV, cytomegalovirus i/e promoter; sd/sa, the SV40 splicing donor/splicing acceptor site; bGH PA, bovine growth hormone polyadenylylation signal sequence. (*B*) Detection of transgenic IL-12 protein in gene gun-treated skin tissues at 24 h after IL-12 cDNA delivery. Plasmid DNA was precipitated onto 2 μm gold particles. Mice were shaved in the abdominal area, and the epidermis was transfected with a 300 psi helium gas pulse by using the helium-pulse Accell device (gene gun). Immunoperoxidase assay demonstrates the presence of IL-12 protein (arrows) in the epidermal cell layers of the test mice. (*C*) Antitumor effect in MethA sarcoma model. Mouse skin overlying and surrounding the target tumor was transfected *in vivo* with IL-12 or Luc cDNA expression vectors on days 7 and 10 after i.d. implantation of 1×10^6 MethA cells. At each treatment, mice received four transfections (5 μg plasmid DNA/treatment). One transfection was directly over the tumor site, and three additional treatments were evenly spaced around the circumference of the tumor in a triangle pattern. Photographs of test mice were taken on days 7, 17, and 29 after tumor cell implantation. Whereas all mice treated with the control (Luc) plasmid DNA developed large tumors, three of eight mice treated with IL-12 gene exhibited complete tumor regression as shown here; the other five mice had reduced tumor growth.

graded numbers of viable effector cells and ^{51}Cr-labeled Renca cells (10^4) were placed into the round-bottomed wells of 96-well plates. After incubating for 4 hr at 37°C, radioactivity in supernatants was determined.

RESULTS AND DISCUSSION

Transgenic Expression of IL-12 *in Vitro* and *in Vivo*. We constructed a plasmid (pWRG3169) containing coding sequences for the p35 and p40 subunits of mIL-12, linked tandomly in the same direction and each driven by its own CMV i/e promoter/enhancer, a SV40 sd/sa intron sequence, and a bovine growth hormone polyadenylylation sequence (Fig. 1*A*). This version of a mIL-12 vector was found to be 3- to 8-fold more efficient in expressing IL-12 protein in B16 tumor cells transfected *in vitro* or in murine skin transfected *in vivo* than the same cDNA clones constructed in a single operon with an internal ribosome entry site linkage. This vector was also more efficient than using gold beads coated with a mixture of two different expression plasmids, one for each IL-12 subunit (data not shown). *In vitro* and *in vivo* expression of IL-12 was performed with this tandom IL-12 gene construct and compared with expression by a control vector containing a Luc cDNA expression plasmid. The level of transgenic IL-12 was determined by a cell proliferation bioassay. Upon *in vitro* gene gun-mediated delivery of 1.25 μg pWRG3169 DNA into 1×10^6 B16 (murine melanoma) cells, 49.8 ± 10.2 ng of functionally active IL-12 were detected at 24 h posttransfection. At 24 h after *in vivo* gene transfer into skin tissue, 266.0 ± 27.8 pg of mIL-12 were detected per 0.172 ± 0.026 g of fresh weight tissue within a standard 1.5×1.5 cm^2 full thickness skin biopsy that contained four gene gun-treated sites. Because of the limited sensitivity of the current IL-12 bioassay for serum and skin tissue extracts (≥100 pg/ml), we were unable to detect the low levels of IL-12 that might have been released into serum of test mice. We have previously shown (14) that very low levels of other cytokines, such as interferon-γ, interleukin 6, or granulocyte/macrophage colony-stimulating factor, can be detected in serum of mice undergoing gene therapy on the skin. It is important to note that the amount of IL-12 detected in the *in vivo* skin transfection sites was 1/400 to 1/40,000 of the dosage (0.1–10 μg) of the systemically injected rIL-12 protein which resulted in both antitumor effects and toxicity in mice (5–7).

Skin tissue overlying a 7-day i.d. Renca tumor was treated with IL-12 expression plasmid by gene gun delivery and biopsied 24 h later. Histologic examination revealed that the gold particles primarily penetrated to the epidermal cell layers of the mouse skin tissue but not into the underlying tumor cells. Accordingly, immunohistochemical staining of the skin tissue 24 h following gene gun delivery with pWRG3169 revealed that transgenic IL-12 was expressed only in the epidermal cell layers (Fig. 1B).

Tumor Regression and Suppression of Tumor Growth Following IL-12 Gene Therapy. It is known that certain murine immunogenic tumors can induce a T cell-mediated immune response that is best detected on days 7–9 of tumor growth in defined tumor models (22–24). Therefore we started the IL-12 cDNA treatments at 7 days postimplantation of tumor cells with the hope of enhancing the already activated endogenous antitumor immune response. Using this experimental strategy, the *in vivo* delivery of the chimeric IL-12 genes into skin tissues overlying established 7-day tumors resulted in complete tumor regression or suppression of tumor growth in four tumor models (Figs. 1C and 2). In mice bearing Renca, L5178Y, MethA, or SA-1 tumors, complete tumor regression was achieved in 87.5% (7/8), 87.5% (7/8), 57% (4/7), and 37.5% (3/8) of the tested mice, respectively (Fig. 2). Nearly identical results were achieved with Renca tumors after a single IL-12 cDNA treatment on day 7 (data not shown). In contrast, tumors grew progressively in most of the untreated mice (data not shown) or mice treated with Luc gene (Fig. 2). Furthermore, in mice bearing P815 mastocytoma or B16 melanoma, a significant suppression of tumor growth was achieved (Fig. 2). For example, on day 13 post-P815 tumor cell implantation, the mean tumor diameter in mice treated with IL-12 gene was 8.89 ± 0.27 mm versus 12.28 ± 0.46 mm in mice treated with Luc control gene in the same expression plasmid ($P < 0.001$). Likewise, on day 15 post-B16 tumor cell implantation, tumor diameter in mice treated with IL-12 gene was 6.30 ± 0.45 mm versus 11.8 ± 0.31 mm in mice treated with Luc gene ($P < 0.001$). However, the observed suppression of tumor growth was transient and all mice eventually died from progressing tumors. Whether or not a modification in gene transfer schedules could improve the result of therapy of these two weakly immunogenic tumors is unclear and warrants further evaluations, especially since most human tumors are believed to be weakly or not immunogenic.

It is important to note that for all tested mouse tumor models, the tumors were already well established at the beginning of the therapy, and had reached 5–8 mm in diameter. To our knowledge, this is the first evidence that an IL-12 gene therapy protocol can cause a complete regression of

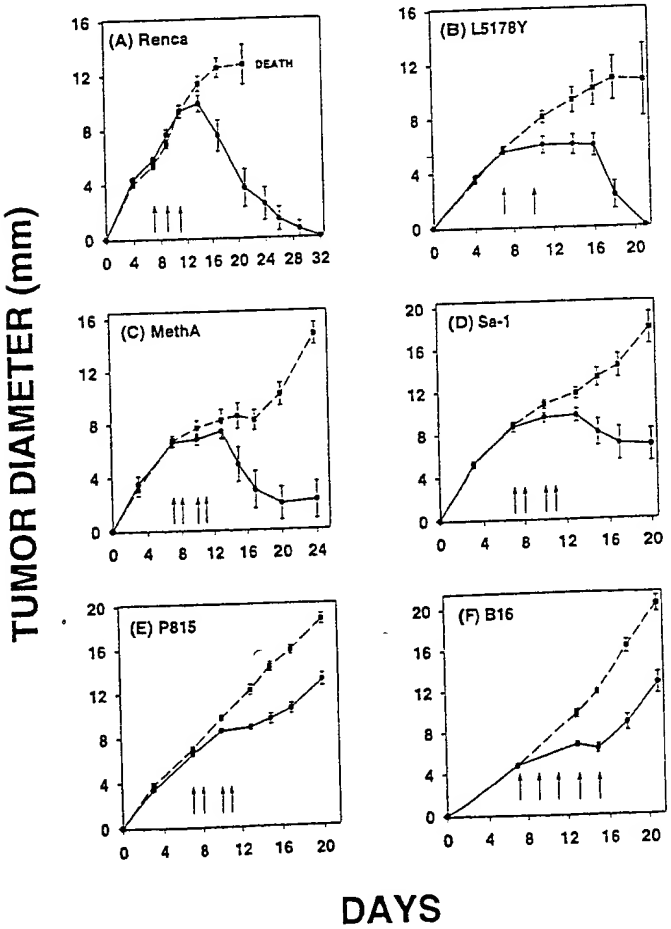

FIG. 2. Kinetics of regression of established murine tumors following *in vivo* IL-12 gene therapy. The gene therapy procedure was started at 7 days after i.d. injection of 1×10^6 (or 1×10^5, in the case of B16 tumor) indicated tumor cells. At each treatment, mice received four transfections with IL-12 DNA (circles) or with control DNA, pCMVLuc (squares). The arrows on each graph indicate the days following tumor injection on which gene transfer treatments were carried out. Mean tumor diameters ± SEM are shown for 7–8 mice per group except for B16 tumor model (12 mice per group). The IL-12 gene therapy experiments were repeated five times with the Renca tumor system, two times with MethA and P815 tumors, and once with L5178Y and B16 tumor models, and similar results were obtained.

established, relatively large tumors. Previous studies have shown that IL-12 gene therapy using retroviral vectors resulted in prevention of tumor development (9), or regression of small, 3-day-old MCA207 sarcomas in 33% of treated mice (10). It is also noteworthy that only 1–4 days of therapy (using four gene gun treatments per tumor site on each day of therapy) resulted in tumor regression or growth suppression in virtually all of our experiments. In previous studies using recombinant protein therapy, tumor regression required daily injections of IL-12 at doses from 0.1 to 10 μg for 1 week (6), or 5 days a week for 3–4 weeks (5) .

It is also important to note that in our IL-12 gene therapy protocol, the normal skin tissue overlying an established tumor is intentionally transfected topically to incite the existing antitumor immune response. The results presented in Figs. 1 and 2, in conjunction with our previous findings using other cytokine genes (14), indicate that transgenic IL-12 production by normal epidermal cells in the vicinity of the tumor is responsible for the antitumor effect of IL-12 gene therapy. Therefore, the *in vivo*, particle-mediated IL-12 gene transfer protocol is drastically different from other currently employed procedures of cancer gene therapy, where a therapeutic vector is either introduced into the tumor or other cells *in vitro*, or injected directly into the tumor mass *in vivo*.

Involvement of CD8$^+$ T Cells in Tumor Regression. To determine the role of CD4$^+$ and CD8$^+$ T cells in the observed tumor regression, we injected Renca tumor-bearing mice with anti-CD4 or anti-CD8 mAb on the next day after the beginning of IL-12 gene therapy and then 4 days later. This protocol was based on our previous findings showing that the same mAbs caused depletion of more than 90% of relevant T cell subsets in mice for 4–5 days following a single injection (25, 26). Fig. 3 provides direct evidence that the IL-12 gene therapy-induced tumor regression required CD8$^+$ cells, in that *in vivo* depletion of CD8$^+$ T cells, but not the depletion of CD4$^+$ T cells, abrogated the effect of IL-12 gene therapy. These data are in agreement with the findings of Brunda *et al.* (5) that tumor regression caused by rIL-12 is mediated by CD8$^+$ T cells, and not by CD4$^+$ T cells. In fact, depletion of CD4$^+$ T cells with anti-CD4 mAb appeared to result in slightly accelerated tumor regression (Fig. 3), implying that CD4$^+$ T cells may have suppressed the anti-tumor effect of IL-12 in this tumor model. Indeed, it has been shown that established tumors induce Th2-like CD4$^+$ T suppressor cells, which can inhibit CD8$^+$ T cell-mediated immune responses (24, 27, 28). The beneficial effect of anti-CD4 mAb treatment for tumor immunotherapy with rIL-2 protein (25) or IL-12 gene (29) has been previously reported. Supporting data show that IL-12 protein can activate tumor-specific CD8$^+$ T cells *in vitro* (30) and mediate an anti-suppressive effect on Th2 CD4$^+$ T cells *in vivo* (31, 32).

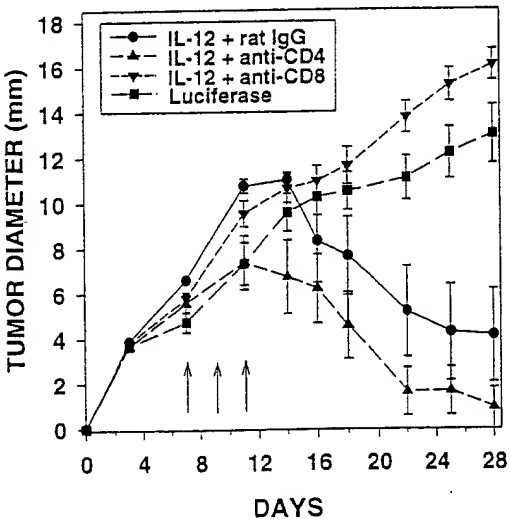

FIG. 3. Tumor regression caused by IL-12 gene requires CD8$^+$ T cells. BALB/c mice were injected i.d. with 1 × 10^6 Renca cells. Skin was transfected with IL-12 or Luc cDNA expression vectors on days 7, 9, and 11 posttumor implantation (arrows). Anti-CD4 mAb (clone GK1.5) or anti-CD8 mAb (clone 2.43), both obtained from the Trudeau Institute (Saranac Lake, NY), were administered intraperitoneally on days 8 (300 μg/mouse) and 12 (150 μg/mouse) after tumor implantation. Control groups included mice that were treated with the IL-12 gene and received rat IgG (Sigma) at the same doses and schedule as the anti CD8-and CD4 mAb, or mice treated with the Luc gene instead of the IL-12 gene. Mean tumor diameters ± SEM are shown for eight mice per group.

Anti-Metastatic Effect of Local IL-12 Gene Therapy. The results showing that tumor regression caused by local IL-12 gene therapy requires CD8$^+$ T cells suggest that local IL-12 gene delivery might result in a systemic antitumor effect. To test this hypothesis, we used the P815 tumor cells that metastasize into the visceral organs several days after the i.d. implantation, thereby causing the death of the mice even when the primary tumor has been surgically removed (20). In this system, an excision of the primary P815 tumor on day 12 (data not shown) or day 15 (Fig. 4) posttumor implantation was followed by death of all treated mice by day 37 or 29, respectively. However, when the skin overlying the i.d. P815 tumors were transfected with IL-12 cDNA on days 12 and 14 posttumor cell implantation, followed by tumor excision on day 15, and three additional IL-12 cDNA skin transfections at the abdominal sites adjacent to the excised primary tumor, a significant prolongation of the survival was observed (Fig. 4). These results suggest that local delivery of IL-12 gene into the skin tissue overlying and surrounding the primary tumor can

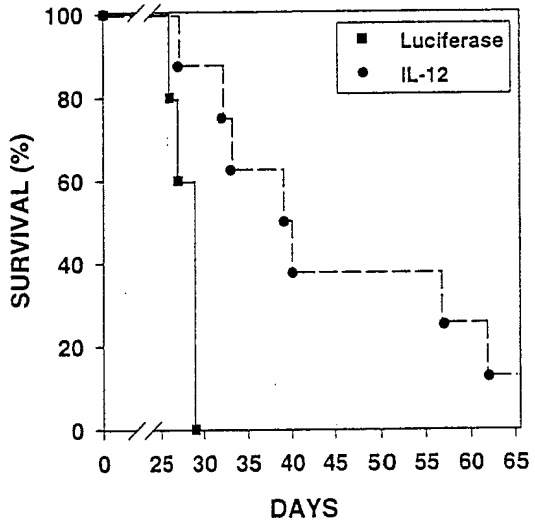

FIG. 4. Anti-metastatic effect of IL-12 gene therapy on P815 tumors. DBA/2 mice were injected i.d. with 1 × 10^6 P815 cells. Skin tissues overlying and surrounding the target tumor were treated with IL-12 cDNA delivered by gene gun (8 mice/group) or Luc cDNA (5 mice/group) on days 12 and 14 after tumor cell implantation. Surgical excision of the tumor was performed as described (20) on day 15, when tumor size reached about 13 mm in diameter. Additional transfections of skin on both sides of the abdomen were performed on days 16, 18, and 20 after implantation of tumor cells. All mice treated with the control Luc cDNA died in 28.0 ± 0.6 days after tumor cell implantation. Death was caused by spontaneous metastases of tumor cells into the internal organs, primarily the liver, as was evidenced by macroscopic examination (data not shown). IL-12 gene therapy effectively prolonged the survival of mice (survival time 41.4 ± 4.9 days, $P < 0.05$), and one of eight mice was "cured." This experiment was repeated without additional transfections posttumor excision, and we observed that all of the Luc cDNA treated mice ($n = 11$) died in 43.9 ± 7.1 days, whereas 5 of 12 (41.6%) IL-12 gene therapy-treated mice survived for at least 180 days and thus were considered cured.

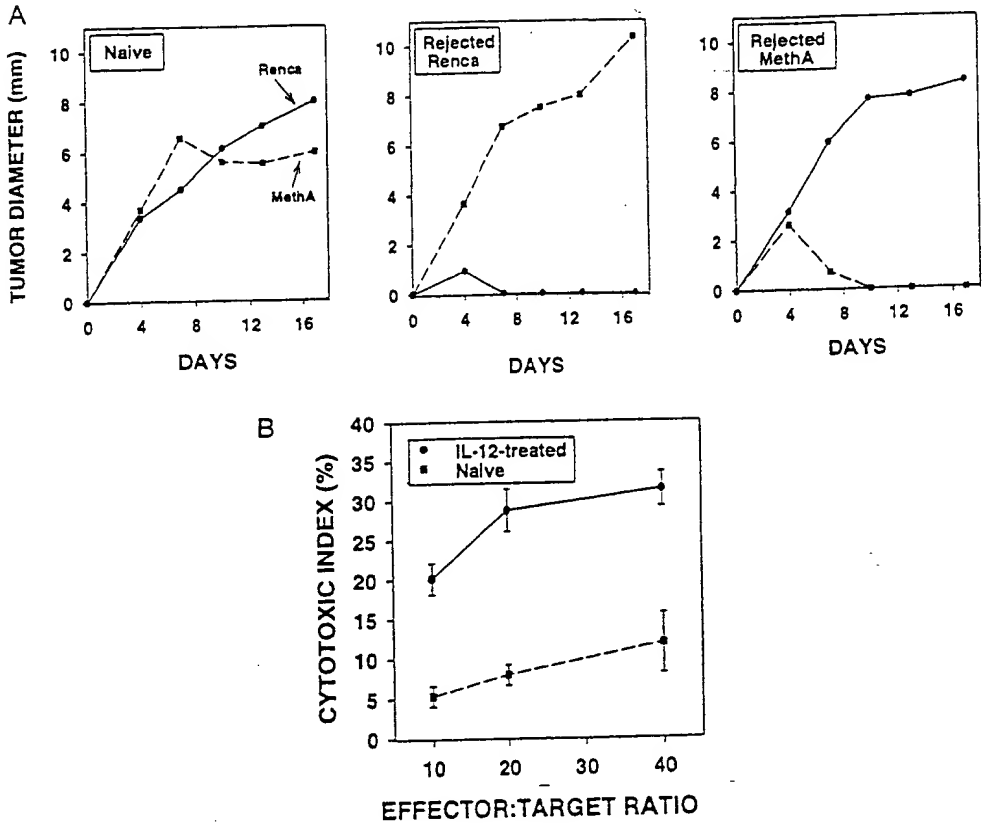

FIG. 5. IL-12 gene therapy in tumor-bearing mice results in development of tumor-specific immunological memory. (*A*) Rejection of secondary tumor challenge following IL-12 gene therapy. BALB/c mice that rejected Renca or MethA tumors following IL-12 gene therapy were injected one month later with 1×10^6 of both Renca cells (circles) and MethA cells (squares) on the right and the left side of abdomen, respectively. As a control, the tumor cells were injected into age-matched naive BALB/c mice. Data are presented as the means of five to eight mice per group. The experiment was repeated using mice that rejected L5178Y tumors and were secondarily challenged with L5178Y or P815 tumor cells, and similar results were obtained. (*B*) Induction of CTL activity in mice that rejected tumors following IL-12 gene therapy. Tumor-specific CTL were generated *in vitro* as described. Mean ± SEM of four mice per group. Spleen cells from IL-12 gene-treated mice generated 3- to 4-fold higher levels of CTL activity than spleen cells from naive mice ($P < 0.005$).

augment systemic antitumor immune response even against a weakly immunogenic tumor, and this can lead to eradication of established spontaneous metastases in mice. Therefore, such human metastatic cancers as subcutaneous T-cell lymphoma or melanoma may provide excellent models for future clinical application of the current IL-12 gene therapy approach.

Immunological Memory in Mice Following IL-12 Gene Therapy. It has been recently shown that tumor regression caused by rIL-12 protein therapy results in development of a memory immune response against the tumor (33). We evaluated if the mice that rejected tumors following the *in vivo* IL-12 gene therapy developed tumor-specific immunity. Fig. 5*A* shows that the mice which rejected Renca tumors and were tumor-free for 1 month resisted a second challenge with Renca cells but developed tumors when challenged with MethA tumor cells. Inversily, the mice that rejected MethA tumors following IL-12 gene therapy resisted the second challenge with MethA cells, but developed tumors when challenged with Renca cells. These results demonstrate that mice which rejected their tumors following skin transfection with IL-12 gene develop tumor-specific immunological memory against a secondary tumor challenge. Furthermore, spleen cells from the mice that rejected Renca tumors, in contrast to spleen cells from naive mice, exhibited CTL activity upon stimulation with Renca cells *in vitro* (Fig. 5*B*). In a similar study, using mice that rejected L5178Y tumors, we found that the generated CTL were tumor-specific, in that they lysed the L5178Y target tumor cells but not the syngeneic P815 target cells (data not shown).

Our study shows that gene gun-mediated *in vivo* delivery of an IL-12 expression plasmid into skin tissue overlying tumor sites is an effective approach for tumor immunotherapy in various murine tumor models, leading to eradication or suppression of established intradermal tumors and their spontaneous metastases. Remarkably, the local amount of detectable IL-12 at the treatment tissue site is 1/400 to 1/40,000 of the dosage of rIL-12 protein employed for efficacy studies in the same or similar mouse tumor models (5, 6, 33). In view of the apparent dose dependent toxicity of human rIL-12 protein in clinical trials (8), this dosage difference between the recombinant protein delivered systemically and transgenic protein produced via *in vivo* DNA delivery may make the present IL-12 gene therapy approach more favorable for clinical considerations. Given the efficacy, simplicity, and potential cost-effectiveness of the gene gun-mediated IL-12 gene therapy approach, further preclinical development of this approach is warranted in order to consider initiation of human cancer clinical trials.

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1. Kobayashi, M., Fitz, L., Ryan, M., Hewick, R. M., Clark, S. C., Chang, S., Koudon, R., Sherman, F., Perussia, B. & Trinchieri, G. (1989) *J. Exp. Med.* **170**, 827–845.

2. Chan, S. H., Perussia, B., Gupta, J. W., Kobayashi, M., Pospisil, M., Young, H. A., Wolf, S. G., Young, D., Clark, S. C. & Trinchieri, G. (1991) *J. Exp. Med.* **173,** 869- 879.
3. Gubler, U., Chua, A. O., Schoenhaut, D. S., Dwyer, C. M., McComas, W., Motyka, R., Mabavi, N., Wolitzky, A. G., Quinn, P. M., Familletti, P. C. & Gately, M. K. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 4143–4147.
4. Wolf, S. F., Temple, P. A., Kobayashi, M., Young, E., Dicig, M., Lowe, L., Dzialo, R., Fitz, L., Ferenz, C., Hewick, R. M., Kelleher, K., Herrmann, S. H., Clark, S. C., Azzoni, L., Chan, S. H., Trinchieri, G. & Perussia, B. (1991) *J. Immunol.* **146,** 3074- 3081.
5. Brunda, M., Luistro, L., Warrier, R., Wright, R., Hubbard, B., Murphy, M., Wolf, S. & Gately, M. (1993) *J. Exp. Med.* **178,** 1223–1230.
6. Nastala, C., Edington, H., McKinney, T., Tahara, H., Nalesnik, M., Brunda, M., Gately, M., Wolf, S., Schreiber, R., Storkus, W. & Lotze, M. (1994) *J. Immunol.* **153,** 1697–1706.
7. Orange, J., Salazar-Mather, T., Opal, S., Spencer, R., Miller, A., McEwin, B. & Biron C. (1995) *J. Exp. Med.* **181,** 901–914.
8. Marshall, E. (1995) *Science* **268,** 1555.
9. Tahara, H., Zeh, H., Storkus, J., Pappo, I., Watkins, S., Gubler, U., Wolf, S., Robbins, P. & Lotz, M. (1994) *Cancer Res.* **54,** 182–189.
10. Tahara, H., Zitvogel, L., Storkus, W., Zeh, H., III, McKinney, T., Schreiber, R., Gubler, U., Robbins, P. & Lotze, M. (1995) *J. Immunol.* **154,** 6466–6474.
11. Yang, N. S. & Sun, W. (1995) *Nat. Med.* **1,** 481–483.
12. Cheng, L., Ziegelhoffer, P. & Yang, N. S. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 4455–4459.
13. Yang, N. S., DeLuna, C. & Cheng, L. (1994) in *Gene Therapeutics: Methods and Applications of Direct Gene Transfer,* ed. Wolff, J. A. (Birkhauser, Boston), pp. 193–209.
14. Sun, W., Burkholder, J., Sun, J., Culp, J., Turner, J., Lu, X., Pugh, T., Ershler, W. & Yang, N. S. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 2889–2893.
15. Desal, B., Quinn, P., Wolitzky, A., Mongini, P., Chizzonite, R. & Gately, M. (1992) *J. Immunol.* **148,** 3125–3132.
16. Zeh, H., III, Hurd, S., Storkus, W. & Lotze, M. (1993) *J. Immunother.* **14,** 155–161.
17. National Institutes of Health (1985) *Guide for Care and Use of Laboratory Animals* (National Institutes of Health, Bethesda, MD), NIH Publ. 86–23.
18. Rajagopalan, L., Burkholder, J., Turner, J., Culp, J., Yang, N. S. & Malter, J. (1995) *Blood* **86,** 2551–2558.
19. Schoenhaut, D., Chua, A., Wolitzky, A., Quinn, P., Dwyer, C., McComas, W., Familletti, P., Gately, M. & Gubler, U. (1992) *J. Immunol.* **148,** 3433–3440.
20. Dye, E. S. (1986) *J. Immunol.* **136,** 1510–1515.
21. Rakhmilevich, A. L., North, R. J. & Dye, E. S. (1993) *Int. J. Cancer* **55,** 338–343.
22. North, R. J. (1984) *Cancer Immunol. Immunother.* **18,** 69–74.
23. North, R. J. & Dye, E. S. (1985) *Immunology* **54,** 47–56.
24. North, R. J. & Bursuker, I. (1984) *J. Exp. Med.* **159,** 1295–1311.
25. Rakhmilevich, A. L. & North, R. J. (1994) *Cancer Immunol. Immunother.* **38,** 107–112.
26. Rakhmilevich, A. L. (1994) *Immunology* **83,** 524–531.
27. Mills, C. & North, R. J. (1985) *Transplantation* **39,** 202–208.
28. Bear, H. D. (1986) *Cancer Res.* **46,** 1805–1812.
29. Martinotti, A., Stoppacciaro, A., Vagliani, M., Melani, C., Spreafico, F., Wysocka, M., Parmiani, G., Trinchieri, G. & Colombo, M. (1995) *Eur. J. Immunol.* **25,** 137–146.
30. Andrews, J. V. R., Schoof, D., Bertagnolli, M., Peoples, G., Goedegebuure, P. & Eberlein, T. (1993) *J. Immunother.* **14,** 1–10.
31. Schmitt, D., Owen-Schaub, L. & Ullrich, S. (1995) *J. Immunol.* **154,** 5114–5120.
32. Nabors, G., Afonso, L., Farrell, J. & Scott, P. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3142–3146.
33. Mu J., Zou J.-P., Yamamoto N., Tsutsui T., Tai X.-G., Kobayashi M., Herrmann S., Fujiwara H. & Hamaoka T. (1995) *Cancer Res.* **55,** 4404–4408.

In Vivo Engineering of a Cellular Immune Response by Coadministration of IL-12 Expression Vector with a DNA Immunogen[1]

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Recent studies support the importance of investigating a DNA vaccination approach for the immunologic control of HIV-1. In this regard, it may be important to specifically engineer immune responses in order to improve on first generation vaccine attempts. Especially for HIV, induction of cell-mediated immunity may be an important feature for any candidate vaccine. In an attempt to engineer in vivo the enhancement of cellular immune response and to direct Ag-dependent immune response from Th2 to Th1 type, we investigated the role of codelivery of genes for IL-12 and granulocyte-macrophage-CSF along with DNA vaccine formulations for HIV-1 Ag. We found that codelivery of IL-12 expression cassettes with DNA vaccines for HIV-1 in mice resulted in splenomegaly as well as a shift in the specific immune responses induced. The codelivery of IL-12 genes resulted in the reduction of specific Ab response, while the coinjection of granulocyte-macrophage-CSF genes resulted in the enhancement of specific Ab response. In addition, we observed a significant Ag-specific stimulation of T cells with codelivery of both cytokines. Most importantly, we observed a dramatic increase in specific CTL response from the group coimmunized with the HIV-1 DNA vaccine and IL-12 genes. This work demonstrates the power of DNA delivery in vivo for both the production of a new generation of more effective and targeted vaccines or immunotherapies as well as an analytic tool for the molecular dissection of the mechanisms of immune function. *The Journal of Immunology,* **1997, 158: 816–826.**

A novel immunization strategy, DNA or genetic vaccination, has been reported to elicit both humoral and cellular immune responses in vivo (1–5). The immune responses are induced upon injection of gene expression cassettes directly into a host target tissue. This delivery of nonreplicating transcription units drives the synthesis of specific foreign proteins within the inoculated host (1–5). These customized synthetic foreign proteins then become the subject of immune surveillance resulting in Ag-specific cellular and humoral immune responses without the associated risk of viral pathogenesis. This vaccination technique is being explored as an effective immunization strategy against a variety of pathogens and, in particular by our group, against HIV-1.

Wang et al. first reported on the production of anti-HIV-1 cellular and humoral immune responses using a DNA vaccine approach. In mice inoculated with HIV-1 gp160 envelope-rev expression vectors by a nucleic acid delivery approach, protective cellular and broad humoral responses were obtained (1, 2, 6). These studies have been extended to nonhuman primates and have demonstrated that protection from SIV/HIV (SHIV) challenge could be achieved in a subset of vaccinated animals in this model (6, 7). Most recently, the ability of this technique to produce immune response(s) in chimpanzees has been reported (7). Furthermore, the first clinical studies of this vaccine approach have started.

Of a spectrum of various host immune responses, induction of cell-mediated immunity could be an especially important requirement in an effective candidate HIV-1 vaccine, because it may play a critical role in viral clearance (8). Cytotoxic lymphocytes target and destroy virus-infected cells by recognizing processed viral fragments presented on the infected cell surface associated with the host-specific MHC class I Ags. CTLs can target not only the gene products present in the viral particle, but also all viral gene products that are expressed during viral replication. Targeting immune responses against viral proteins through the development of specific CTL responses could aid in lowering viral load through destruction of viral factories, thus lowering the establishment of initial viral load.

While the above studies support the importance of pursuing a DNA vaccination approach for HIV-1, it may be important to specifically direct immune responses in order to improve on first-generation vaccine attempts. In this regard the type of immune response induced has been suggested to be important in HIV pathogenesis (9). The type of immune response (Th1 vs Th2) has been reported to be important in a variety of disease models including infectious diseases, autoimmune diseases, and allergies (10–15). To induce strong and stable cell-mediated immune response against HIV infection, the use of immunologic adjuvants and immune modulators such as cytokines in conjunction with immunization could enhance cellular immune response and direct Ag-dependent immune response from Th2 to Th1 type.

In an attempt to engineer the immune response in vivo we investigated the role of codelivery of cytokine genes along with HIV

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constructs. We examined responses induced by granulocyte-macrophage-CSF (GM-CSF)[4] codelivery, as well as the Th1-inducing cytokine IL-12, along with DNA vaccine formulations for HIV-1 on the induction of cellular immunity, specifically anti-viral CTL responses. IL-12 is a cytokine that plays a critical role in Th1 immune response mainly by inducing production of Th1-associated cytokine IFN-γ. It activates NK and T cells through induction and release of various cytokines including IFN-γ (16–19).

GM-CSF is a hemopoietic growth factor that stimulates neutrophil, monocyte/macrophage, and eosinophil colony formation (20–23). It also induces proliferation and differentiation of erythroid and megakaryocyte progenitor cells (20–23). GM-CSF also increases the Ab-dependent cell-mediated cytotoxicity of neutrophils, eosinophils, and macrophages but has not been reported to have a direct role in the generation of CTL response in vivo (22).

The genes for murine IL-12 and murine GM-CSF were individually cloned into expression vectors under control of a CMV promoter. The gene plasmid expression cassettes were then injected into mice along with DNA vaccine cassettes for HIV-1, which have been described previously (6, 7). We analyzed the immunologic effects of the coimmunization with these genetic adjuvant cassettes on the magnitude of Ag-specific immune responses. A reduction in humoral response was seen with IL-12 codelivery while an enhancement of humoral response was seen with GM-CSF coimmunization. An increase in Ag-specific Th cell proliferation was seen with either IL-12 or GM-CSF coimmunization. Importantly, we observed a significant enhancement of CTL response in vivo with the coadministration of murine IL-12 genes with four different HIV-1 DNA immunogens (gag/pol, envelope, vif, and nef). In contrast, almost no effect on CTL induction was observed with the genes for GM-CSF in these studies. These results demonstrate the utility of DNA vaccines for the tailored production of specific immune responses. They also demonstrate the utility of this approach to elucidate basic immunologic functions in a molecule-specific fashion.

Materials and Methods

Mice

BALB/c female mice, aged 6 to 8 wk, were purchased from Harlan Sprague-Dawley, (Indianapolis, IN). The mice were housed in a temperature-controlled, light-cycled room. Their care were under the guidelines of the National Institutes of Health (Bethesda, MD) and the University of Pennsylvania (Philadelphia, PA).

Reagents

DNA vaccine constructs pCEnv and pCGag/Pol were prepared as previously described (6, 7). Murine IL-12 (p35 and p40 chains) and GM-CSF genes were received from W. M. F. Lee and were cloned into pCDNA3 expression vector (Invitrogen, San Diego, CA). *Nef* and *Vif* genes were cloned into pCDNA3.[5] Recombinant vaccinia (vMN462, vVK1, VV:gag, and vSC8) were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. Nef peptide 73–82 was synthesized according to the previously published protocol (24, 25).

DNA inoculation

We have utilized a facilitated DNA inoculation protocol that results in increased protein expression levels from plasmid-delivered genes in vivo. Specifically, the quadriceps muscles of BALB/c mice were injected with 100 μl of a solution containing 0.25% bupivacaine-HCl (Sigma Chemical Co., St. Louis, MO) using a 27-gauge needle (1, 2). Two days later, 50 μg of each DNA construct of interest in PBS was injected into the same region of the muscle as the bupivacaine injection. Coadministration of various gene expression cassettes involved mixing the chosen plasmids prior to injection.

FACS analysis

Cells (1×10^5) were washed three times with FACS buffer (PBS containing 1% BSA and 0.1% sodium azide) and incubated with FITC- and/or phycoerythrin-conjugated mAbs at a saturating condition for 30 min on ice. After being washed three times with FACS buffer, cells were analyzed using a FACScan (Becton Dickinson, Mountain View, CA).

ELISA

Fifty microliters of gp120 or p24 (Intracel Corporation, Cambridge, MA) in 0.1 M carbonate-bicarbonate buffer, pH 9.5, at 2 μg/ml concentration was adsorbed onto microtiter wells overnight at 4°C as previously described. The plate was washed with PBS-0.05% Tween-20 and blocked with 3% BSA in PBS with 0.05% Tween-20 for 1 h at 37°C. Mouse antisera were diluted with 0.05% Tween-20 and incubated for 1 h at 37°C, then incubated with a manufacturer-suggested dilution of horseradish peroxidase-conjugated goat anti-mouse IgG or IgA (Sigma). The plate was washed and developed with TM blue buffer (Sigma). The OD 450 nm was read on a Dynatech MR5000 plate reader.

Th cell proliferation assay

Lymphocytes from harvested mouse spleens were prepared. The isolated cell suspensions were resuspended to a concentration of 1×10^6 cells/ml. A 100-μl aliquot containing 1×10^5 cells was immediately added to each well of a 96-well microtiter flat-bottom plate. One hundred microliters of protein (p55) was added to wells in triplicate at 20 μg/ml, making the final protein concentration at 10 μg/ml. The cells were incubated at 37°C in 5% CO_2 for 3 days. One microcurie of tritiated thymidine was added to each well, and the cells were incubated for 12 to 18 h at 37°C. The plate was harvested, and the amount of incorporated tritiated thymidine was measured in a Beta Plate reader (Wallac, Turku, Finland). To assure that cells were healthy, 10 μg/ml of PHA was used as a polyclonal stimulator-positive control. The stimulation index was determined from the formula: Stimulation index = (experimental count − spontaneous count)/spontaneous count.

CTL assay

A 5-h ^{51}Cr release CTL assay was performed using vaccinia-infected targets or peptide-treated targets. Lymphocytes were harvested from spleens and prepared as the effector cells by removing the erythrocytes and by washing several times with fresh media. The assay was performed both with and without in vitro stimulation of the effectors. In the case of in vitro stimulated assay, the effector cells were stimulated for 2 days with Con A at 2-μg/ml concentration. Then the effectors were stimulated either with relevant vaccina-infected cells, which were fixed with 0.1% glutaldehyde, or with relevant peptides at the 1 μM concentration for 3 to 4 days. Vaccinia-infected targets were prepared by infecting 3×10^6 p815 cells for 5 to 12 h at 37°C. Peptide-treated targets were prepared by incubating p815 cells for 2 h with peptides. The target cells were labeled with 100 μCi/ml $Na_2{}^{51}CrO_4$ for 60 to 120 min and used to incubate the stimulated splenocytes for 4 to 6 h at 37°C. CTL was tested at E:T ratios ranging from 50:1 to 12.5:1. Supernatants were harvested and counted on an LKB CliniGamma gamma counter. The percentage of specific lysis was determined from the formula:

$$100 \times \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}}.$$

Maximum release was determined by lysis of target cells in 1% Triton X-100-containing medium. An assay was not considered valid if the value for the "spontaneous release" counts are in excess of 20% of the "maximum release".

Results

To enhance the magnitude of cellular immune response to DNA vaccines in vivo, we investigated coadministration of immunologically important cytokine genes. Several cytokines have been directly implicated in expression of cellular responses in vivo. Of those cytokines, IL-12 is thought to be very important (26). Additionally, GM-CSF has been reported to be important in in vivo tumor and viral protection studies (27, 28). Accordingly, we tested a stimulation of immune responses by both cytokine modulators along with HIV gene vaccines.

[4] Abbreviations used in this paper: GM-CSF, granulocyte-macrophage colony-stimulating factor; SHIV, simian immunodeficiency virus/human immunodeficiency virus.

[5] Ayyavoo, V. 1997. Development of genetic vaccines for pathogenic genes: construction of attenuated Vif DNA immunization cassettes. *Submitted for publication.*

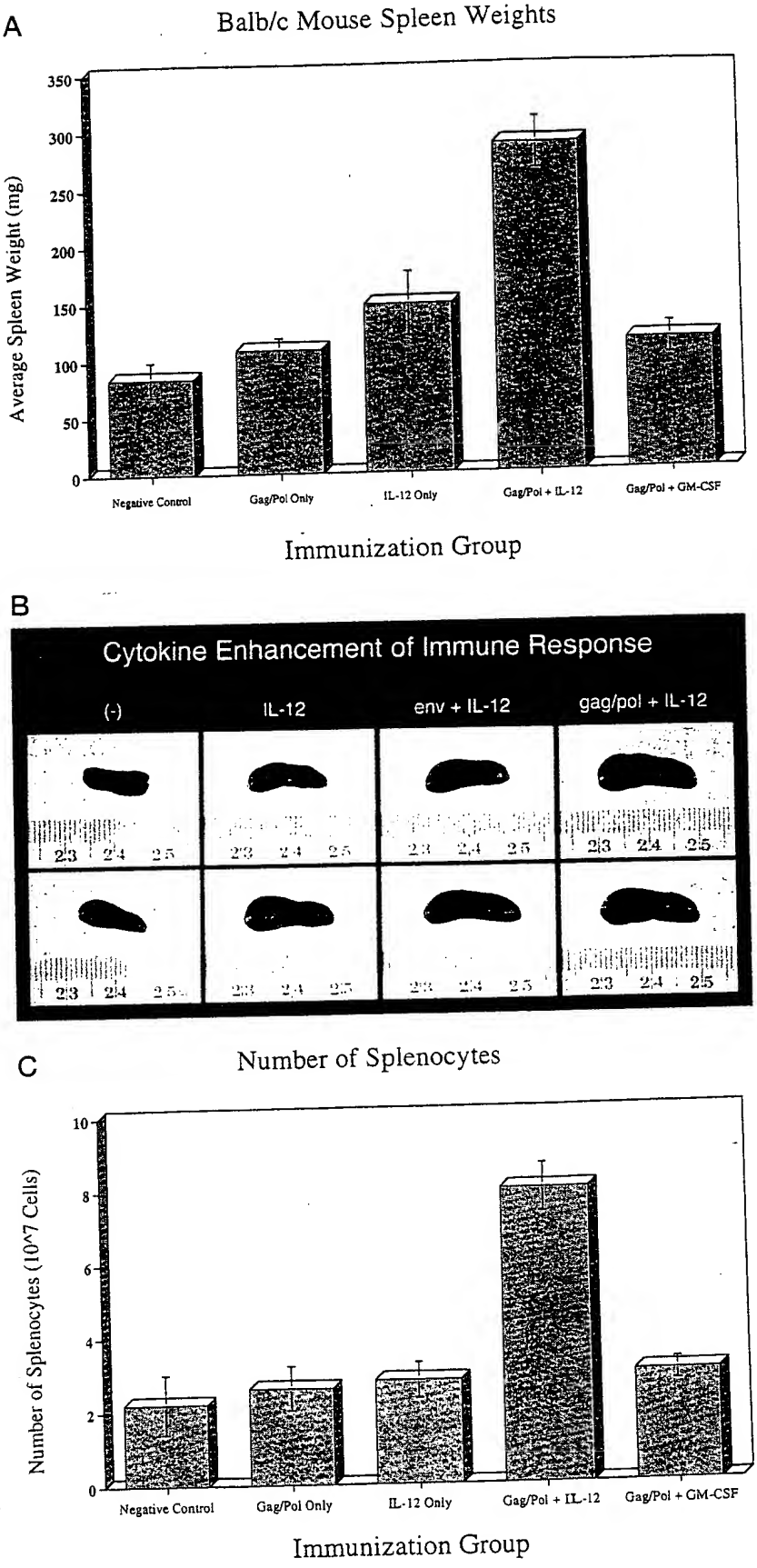

FIGURE 1. *A,* Fifty micrograms of respective cDNA expression cassettes were administered i.m. at day 0. At 14 days after immunization spleens were harvested from all immunized animals and weighed. The negative control animals were unimmunized. The weights of the spleens from the mice injected with pCGag/pol or IL-12 genes alone was similar to those of the unimmunized control mice (about 100 mg). However, the spleens from mice injected with pCGag/pol and IL-12 genes weighed almost three times as much as the control spleens. In contrast, pCGag/pol- and GM-CSF-immunized mouse spleens were not enlarged. Each group included at least eight mice. *B,* Harvested spleens were photographed. The visual size of the spleens corresponded directly to the weights in which the DNA immunogen-IL-12-vaccinated spleens were significantly larger than the unimmunized control spleens. Groups: (−) unimmunized; IL-12 immunized; pC-Env + IL-12 immunized; pCGag/pol + IL-12 immunized. *C,* The splenocytes were prepared and purified from each spleen. Corresponding directly to their spleen weight difference, the number of cells from the pCGag/pol + IL-12-immunized spleens were more than three times the cell number derived from the control spleens. pCGag/pol + GM-CSF-immunized mouse spleens did not have any significant increase in the number of lymphocytes above the control spleen cell number.

Phenotyping of mouse spleens

Following coinoculation, it was observed that spleens collected from individual experimental groups appeared different. Accordingly, spleens collected from all immunized animals were weighed and visually examined. The spleen weights of these animals are shown in Figure 1*A*. Although the weights of the spleens of the mice injected with single formulation controls were similar to those of the unimmunized control mice (about 100 mg), the

Table I. *Percentage of cell population in spleen (by FACS analysis)*

	Unimmunized	Immunized with Env + IL-12	Immunized with Gag/Pol + IL-12
$CD3^-B220^+$	25.43	17.46	21.62
$CD3^+CD4^+$	34.51	39.52	32.08
$CD3^+CD8^+$	13.69	21.72	16.88

spleens from mice injected with pCGag/pol + IL-12 genes weighed about three times as much as the control spleens. On the other hand, pCGag/pol + GM-CSF-immunized mouse spleens were not enlarged. It is interesting to note that those immunized with pCGag/pol only or IL-12 alone did not result in significantly enlarged spleens. Only when the Ag and IL-12 gene cassettes were coinjected did the splenomegaly result, suggesting that this was a combined effect of both gene products. The representative spleens are shown in Figure 1*B*. Corresponding to their weights, we observed the Ag + IL-12 spleens to be several times larger than other spleens. Furthermore, as shown in Figure 1*C*, the number of lymphocytes derived from the pCGag/pol + IL-12 spleens were more than three times the number derived from the control spleens. Again, the pCGag/pol + GM-CSF immunized mouse spleens did not have any significant increase in the number of lymphocytes above the control spleen cell number.

FACS analysis

To further characterize the cellular composition of the enlarged spleens, we performed FACS analysis. Table I shows the FACS results from the double staining of the splenocytes with Abs for CD3 with Abs for B220, CD4, and CD8. As shown, we observed a slight reduction in the percentage of B220-positive B cell population in the groups immunized with pCEnv + IL-12 or pCGag/pol + IL-12 constructs (17.46 and 21.62%, respectively) from the percentage of B cell in unimmunized control spleens (25.43%). In addition, there was a moderate increase in the percentage of $CD8^+$ T cells in the groups immunized with pCEnv + IL-12 or pCGag/pol + IL-12 constructs (21.72 and 16.88%, respectively) vs the percentage in the unimmunized group (13.69%).

Humoral response

Antisera from immunized mice were collected and analyzed for specific Ab responses against HIV-1 Ags by ELISA. Figure 2 shows the ELISA results from the samples collected at 14 and 28 days after immunization. At 1:100 dilution, sera from the group immunized with pCGag/pol + GM-CSF showed Ab response against HIV-1 p24 protein, which was greater than those of the group immunized with pCGag/pol alone. On the other hand, the group immunized with pCGag/pol + IL-12 showed a significantly less humoral response over the same period. Similarly, those mice immunized with pCEnv + GM-CSF had Ab response against HIV-1 gp120 protein (at 1:100 dilution) that was greater than those of the group immunized with only pCEnv or pCEnv + IL-12 over the same period (Fig. 3). Again, the group immunized with pCEnv + IL-12 showed a significantly less humoral response over the period. In repeated experiments, IL-12 generally suppressed specific Ab responses by 10 to 20%, while GM-CSF appeared to have the opposite effect. This humoral effect could be related to the observed change in B cell number in the splenocytes as identified on FACS analysis.

Th cell proliferation assay

Activation and proliferation of Th lymphocytes play a critical role in inducing both humoral immune response via expansion of Ag-activated B cells and cellular immune response via expansion of $CD8^+$ cytotoxic T lymphocytes. Two weeks after the first DNA immunization, the mice were boosted with the same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated. These cells were then tested for T cell proliferation as described above. Figure 4 shows the proliferation assay results for the mice immunized with DNA vaccine encoding for HIV-1 gag/pol (pCGag/pol) and those mice co-immunized with pCGag/pol and IL-12 or GM-CSF. Recombinant p55 protein (10 μg/ml) was plated in each well to stimulate proliferation of T cells. A total of 10 μg/ml of lectin PHA was used as a polyclonal stimulator positive control. As shown, a low background level of proliferation was observed from a control group of naive mouse spleens with a stimulation index of 1.2, and a moderate level of proliferation was observed from the

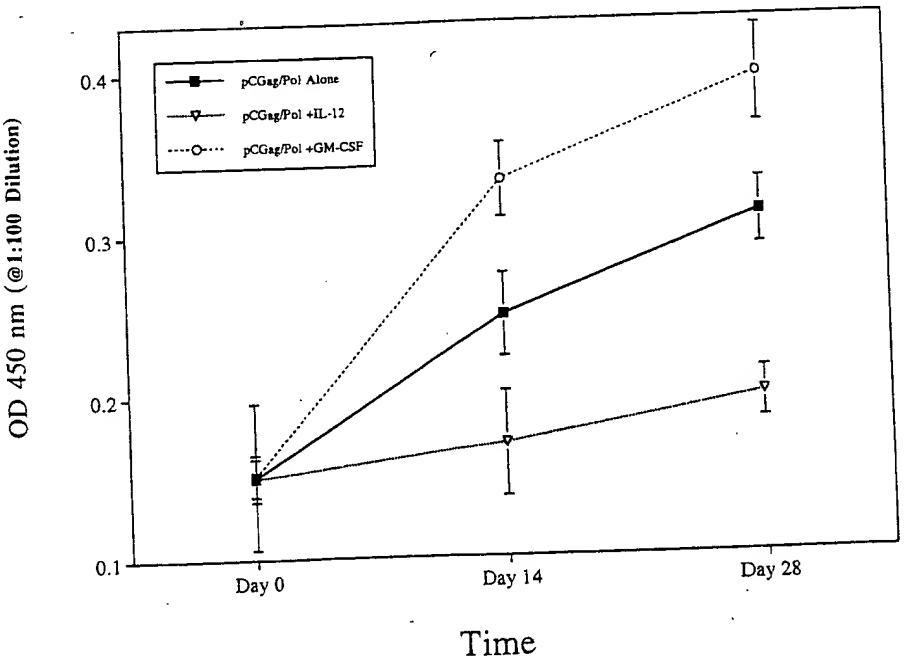

FIGURE 2. Fifty micrograms of IL-12 or GM-CSF genes were coadministered with pCGag/pol cDNA expression cassettes i.m. at day 0. Prior to injection and at days 14 and 28, the mice were bled and the sera collected. The mouse serum was tested for Gag/pol-specific Ab response using the ELISA as described using HIV-1 p24 protein. At 1:100 dilution, the pCGag/pol + GM-CSF-immunized groups had specific Ab response that was significantly greater than those of pCGag/pol-immunized group. In contrast, pCGag/pol + IL-12 group had significantly less Ab response against p24.

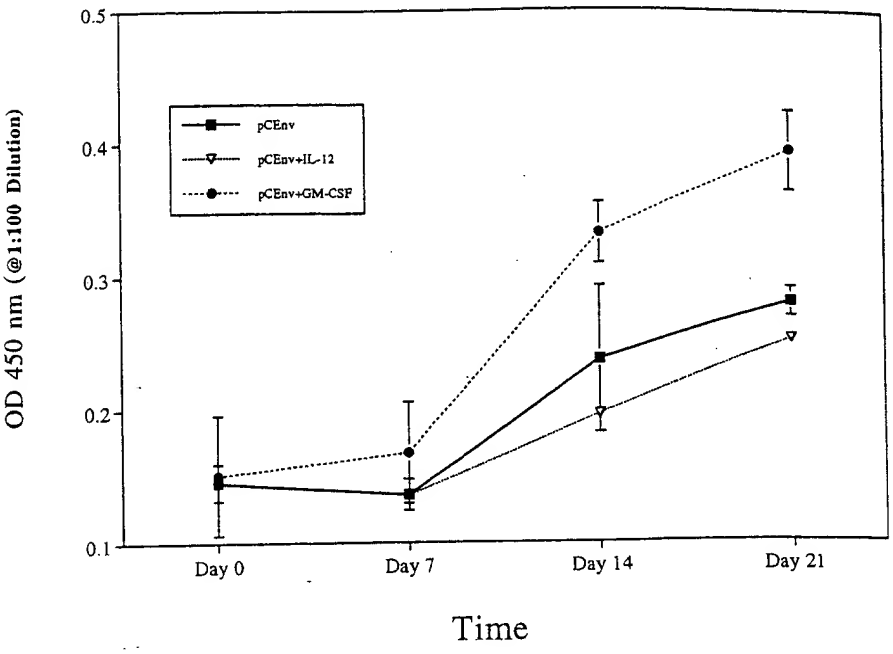

FIGURE 3. Fifty micrograms of IL-12 or GM-CSF genes were coadministered with pCEnv cDNA expression cassettes i.m. at day 0. Prior to injection and at days 7, 14, and 21, the mice were bled and the sera collected. The mouse serum was tested for envelope-specific Ab response using the ELISA as described using HIV-1 gp120 protein. Similar to the results in Figure 2, at 1:100 dilution the pCEnv + GM-CSF immunized groups had specific Ab response that was significantly greater than those of pCEnv-immunized group. Again, pCEnv + IL-12 group had significantly less Ab response against gp120. In repeated experiments, IL-12 generally suppressed specific Ab responses by 10 to 20% while GM-CSF had the opposite effect.

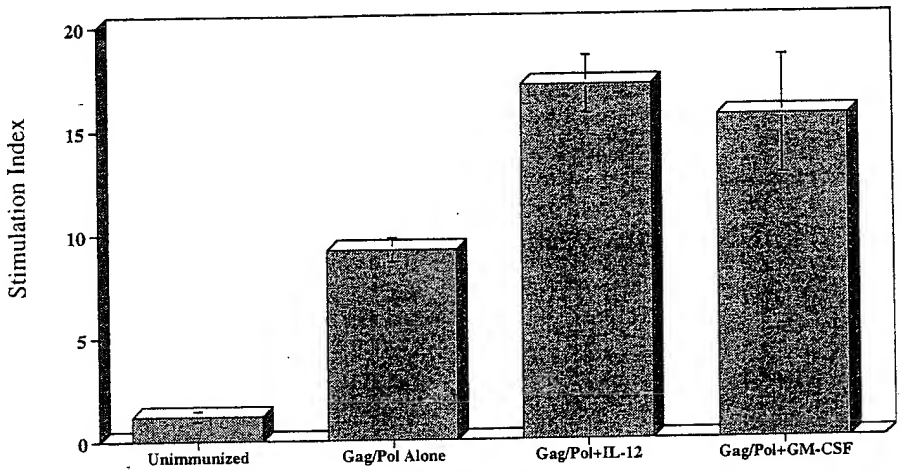

FIGURE 4. Activation and proliferation of Th lymphocytes play a critical role in inducing both humoral immune response via expansion of Ag-activated B cells and cellular immune response via expansion of $CD8^+$ cytotoxic T cells as well as playing a crucial role in Ag-specific immune responses. Fifty micrograms of respective cDNA expression cassettes were administered i.m. at day 0. Two weeks after the first DNA immunization, the mice were boosted with the same dosage. After one additional week, spleens were collected from immunized mice and their lymphocytes were isolated. These cells were then tested for T cell proliferation as described above. Figure 4 shows the proliferation assay results for the mice immunized with DNA vaccine encoding for HIV-1 gag/pol (pCGag/pol) and those mice coimmunized with pCGag/pol and IL-12 or GM-CSF. Recombinant p55 protein (10 μg/ml final concentration) was plated in each well to stimulate the proliferation of Th cells. Ten micrograms per milliliter of lectin PHA were used as a polyclonal stimulator-positive control. As shown, a low background level of proliferation was observed from the control group of naive mouse spleens with a stimulation index of 1.2, and a moderate level of proliferation was observed from the group immunized with pCGag/Pol alone with a stimulation index of 9.2. A significantly increased level of proliferation was seen from group coimmunized with pCGag/pol + IL-12 genes with a stimulation index of 17.1 and a group coimmunized with pCGag/pol + GM-CSF genes with the stimulation index of 15.6. The stimulation index of PHA-stimulated control was 58.8.

group immunized with pCGag/pol alone with a stimulation index of 9.2. A dramatic boosting in the proliferation was seen from the group coimmunized with pCGag/pol and IL-12 genes with a stimulation index of 17.1 and the group coimmunized with pCGag/pol and GM-CSF genes with a stimulation index of 15.6.

Ag-specific cytotoxic T lymphocyte activity

To further investigate the enhancement of the cellular activity, we conducted CTL assays on splenocytes of mice immunized with pCGag/pol, pCEnv, pCVif, and pCNef. The CTL assay was performed on spleen cells harvested from immunized mice both with and without in vitro stimulation of the spleen cells prior to the assay measuring the chromium release from specific and nonspecific vaccinia-infected or peptide-treated targets. To find specific lysis of targets, the percentage of lysis of irrelevant targets was subtracted from the percentage of lysis of specific targets.

Gag/pol

Two weeks after the first DNA immunization, the mice were boosted with the same dosage. After one additional week, spleens were collected from immunized mice and their lymphocytes were isolated. The CTL assay was performed on these

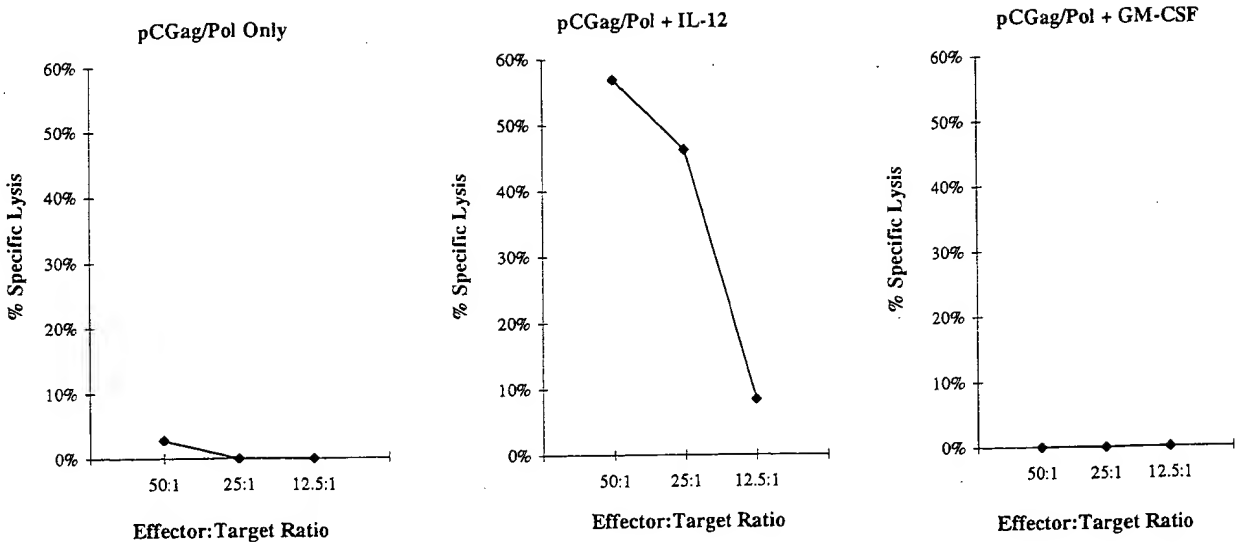

FIGURE 5. Fifty micrograms of IL-12 or GM-CSF genes were coadministered with pCGag/pol cDNA expression cassettes i.m. Two weeks after the first DNA immunization, the mice were boosted with same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated. The CTL assay was performed on spleen cells harvested from immunized mice as described above with no in vitro stimulation induced on the splenocytes. The assay was conducted on the day of spleen harvest, measuring the chromium release from specific and irrelevant vaccinia-infected targets. The control group immunized with only IL-12 gene cassette resulted in no specific lysis of target cells above the background level. In addition, low level (3%) of specific lysis was observed with pCGag/pol-only immunization at the 50:1 E:T ratio. In contrast, 57% specific lysis was seen with pCGag/pol + IL-12 coadministration samples at the 50:1 E:T ratio and titered out to 8% at the 12.5:1 E:T ratio. Those immunized with pCGag/pol + GM-CSF plasmids resulted in no detectable CTL activity. Recombinant vaccinia vVK1 and vSC8 were used to infect p815 to prepare specific and irrelevant targets, respectively.

cells without in vitro stimulation induced on the splenocytes. The assay was conducted on the day of spleen harvest measuring the chromium release from specific and nonspecific vaccinia-infected targets. We observed a dramatic increase in specific CTL activity from Gag/pol + IL-12-immunized splenocytes (Fig. 5). The control group immunized with only IL-12 gene cassette resulted in no specific lysis of target cells above the background level. In addition, low level (3%) of specific lysis was observed with Gag/pol-only immunization at the 50:1 E:T ratio. In contrast, 57% specific lysis was seen with Gag/pol + IL-12 coadministration samples at the 50:1 E:T ratio and titered out to 8% at the 12.5:1 E:T ratio. On the other hand, those immunized with Gag/pol and GM-CSF plasmids resulted in no detectable CTL activity.

Envelope

Similar results were observed from the mice coimmunized with the pCEnv construct and cytokine genes (Fig. 6). Two weeks after the first DNA immunization, the mice were boosted with the same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated. In a CTL assay performed with in vitro stimulation, the groups immunized with pCEnv alone and pCEnv + GM-CSF resulted in low levels of specific CTL at 4% and 2%, respectively. On the other hand, a dramatically enhancement of CTL activity was again observed in the pCEnv + IL-12 group at 53% lysis.

Vif

HIV-1 accessory proteins are believed to play a major role in HIV pathogenesis and have been proposed as potential targets for candidate HIV-1 vaccines (6). We immunized mice with DNA expression cassettes encoding the accessory proteins vif (pCVif) and nef (pCNef). Fifty micrograms of IL-12 genes and pCVif were coadministered i.m. The mice were boosted with the same dosage at week 2 and week 4 of post-immunization. In CTL assays performed on spleen cells harvested from mice immunized with pCVif constructs without in vitro stimulation, we observed a dramatic increase in specific CTL response with IL-12 gene coadministration (Fig. 7). At the 50:1 E:T ratio, a moderate level (17%) of specific lysis

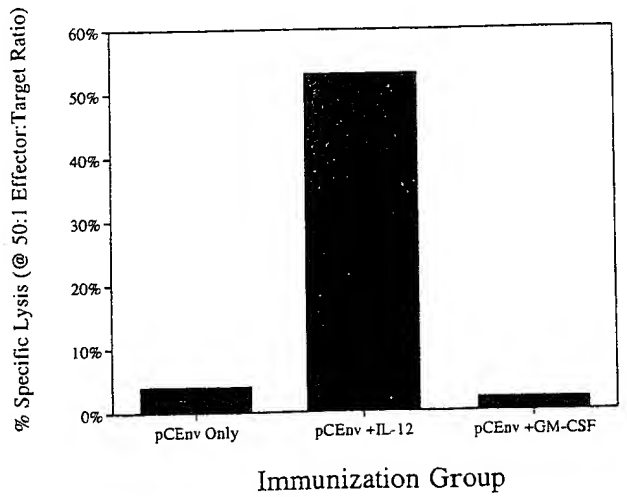

FIGURE 6. Fifty micrograms of IL-12 or GM-CSF genes were coadministered with pCEnv cDNA expression cassettes i.m. Two weeks after the first DNA immunization, the mice were boosted with same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated. The CTL assay measuring the chromium release from specific and irrelevant vaccinia-infected targets was performed on spleen cells harvested from immunized mice with in vitro stimulation induced on the splenocytes. At 50:1 E:T ratio, the group immunized with pCEnv alone and pCEnv + GM-CSF resulted in a low levels of specific CTL at 4% and 2%, respectively. On the other hand, a dramatic enhancement of CTL activity was seen from the pCEnv + IL-12 immunized group at 53%. Recombinant vaccinia vMN462 and vSC8 were used to infect p815 to prepare specific and irrelevant targets, respectively.

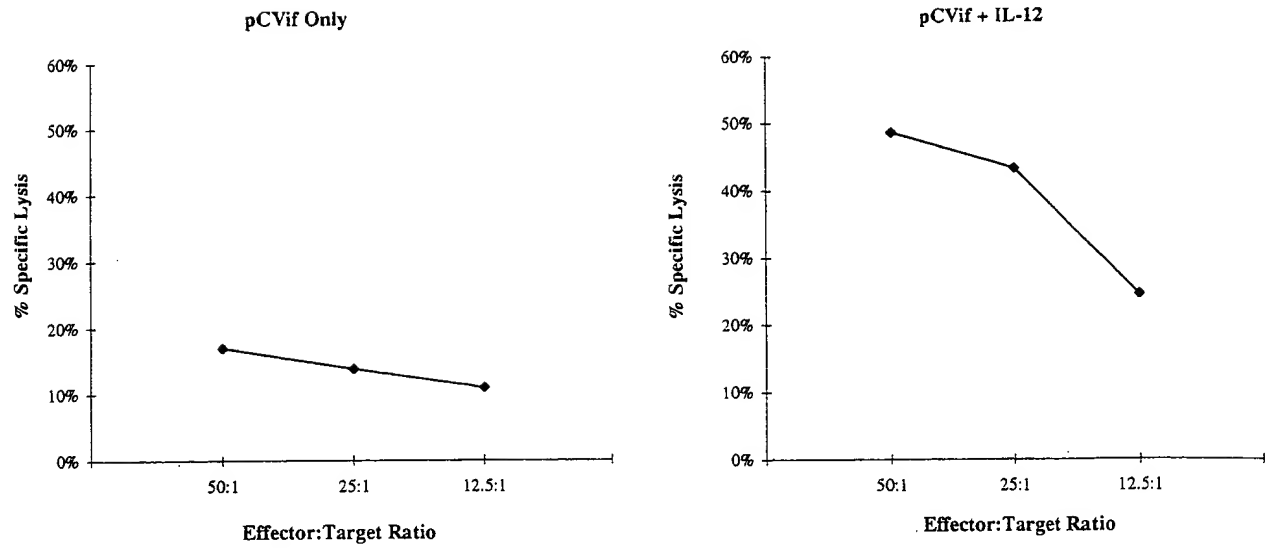

FIGURE 7. Fifty micrograms of IL-12 genes were coadministered with pCVif cDNA expression cassettes i.m. The mice were boosted with the same dose at week 2 and week 4 after immunization. Spleens were collected from immunized mice and their lymphocytes were isolated. The CTL assay measuring the chromium release from specific and irrelevant vaccinia-infected targets was performed on spleen cells harvested from immunized mice as described above with no in vitro stimulation induced on the splenocytes. At the 50:1 E:T ratio, a moderate level (17%) of specific lysis was observed with the group immunized with pCVif. In contrast, 49% specific lysis was seen with pCVif + IL-12 coadministration at the same E:T ratio. The results titered out to 11% specific lysis for the pCVif-inoculated group at the 12.5:1 E:T ratio whereas the pCVif + IL-12 group resulted in 25% specific lysis of the targets. Recombinant vaccinia VV:gag and vSC8 were used to infect p815 to prepare specific and irrelevant targets, respectively.

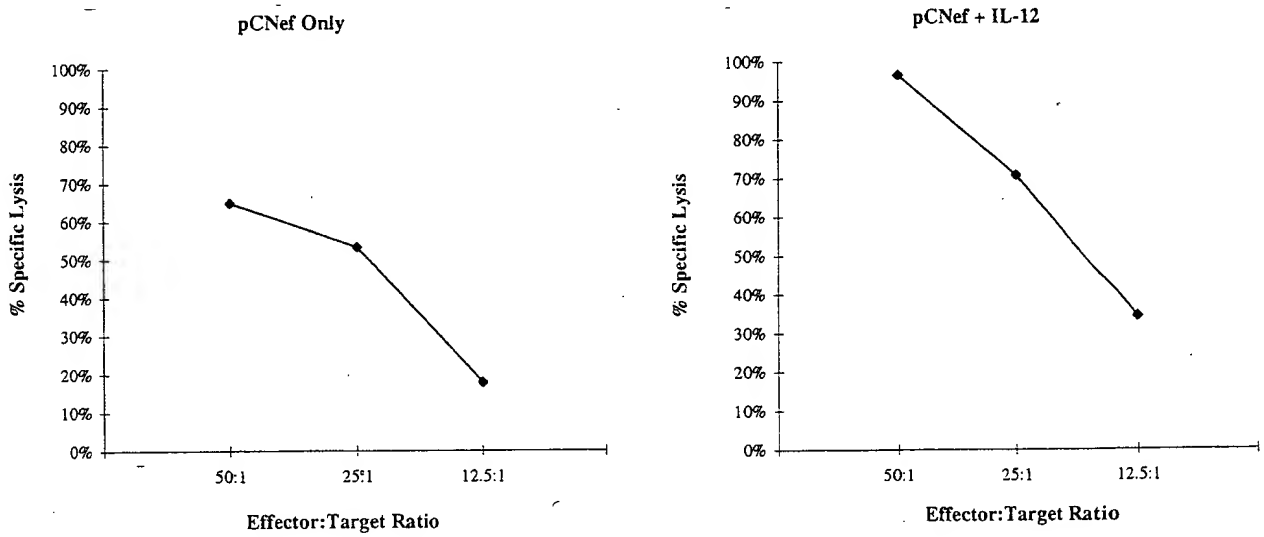

FIGURE 8. Fifty micrograms of IL-12 genes were coadministered with pCNef cDNA expression cassettes i.m. The mice were boosted with same dosage at week 2. Spleens were collected from immunized mice and their lymphocytes were isolated. A CTL assay with nef peptides (peptide 71–83) on the spleen cells harvested from the pCNef-immunized groups. The nef peptide chosen was published to be MHC class I restricted as well as $CD8^+$ dependent. At the 50:1 E:T ratio, 65% specific lysis was observed with the group immunized with pCNef. In contrast, 97% specific lysis was seen with pCNef + IL-12 coadministration at the same E:T ratio. The results titered out to 18% specific lysis for the pCNef-inoculated group at the 12.5:1 E:T ratio whereas the pCNef + IL-12 group resulted in 34% specific lysis of the targets. The coimmunization of pCNef with IL-12 genes resulted in the increase in the specific CTL activity by 17 to 23% at various E:T ratios. In every DNA immunogen experiment (Figs. 5 through 8), the same CTL assay conducted against irrelevant targets did not result in any significant CTL response. These results indicate that the dramatic enhancement of CTL activity from Ag and IL-12 DNA immunization were not due to an enhancement of NK activity as the results were Ag specific and T cell dependent.

was observed with the group immunized with pCVif. In contrast, 49% specific lysis was seen with pCVif + IL-12 coadministration at the same E:T ratio. The results titered out to 11% specific lysis for the pCVif inoculated group at the 12.5:1 E:T ratio whereas the pCVif + IL-12 group resulted in 25% specific lysis of the targets.

Nef

Two weeks after the first pCNef DNA immunization, the mice were boosted with the same dosage. After 1 additional week, spleens were collected from immunized mice and their lymphocytes were isolated. We performed a CTL assay with nef peptides on these cells. The nef peptide chosen (peptide 71–83) is well characterized and is reported to be MHC class I restricted as well as $CD8^+$ dependent (29–31). We observed that pCNef immunization resulted in a significant CTL response (Fig. 8). Even more, we found that the coinoculation of pCNef with IL-12 genes elicited an enhancement of specific lysis of targets. In fact, the coimmunization of pCNef with IL-12 genes resulted in the increase in the specific CTL activity by 17 to 23% at various E:T ratios. In every DNA immunogen experiment (Figs. 5 through 8), the same CTL

assay conducted against irrelevant targets did not result in any significant lysis of the target cells. These results indicate that the consistent and dramatic enhancement of CTL activity seen from Ag and IL-12 DNA immunization were not due to an enhancement of NK activity, since the results were Ag specific and T cell dependent.

Requirement of both IL-12 chains for in vivo effects

It has been reported that p35 chain of IL-12 is constitutively expressed in many cell types (26). Therefore it may be possible that only a single p40 chain and DNA immunogen were responsible for the effects. To test this directly in vivo, we coadministered each of the two IL-12 heterodimer genes (p35 and p40) with pCGag/pol. As shown in Figure 9*A*, we did not observe any enlargement of spleen size in either case. These data indicate that the coinjection with DNA vaccine and both p35 and p40 IL-12 genes resulted in the increased size of spleen and corresponding augmentation of the number of splenic cells. These data support that the plasmids probably entered the same cells in vivo and coordinated transcription of all three components, p35 chain, p40 chain, and the specific Ag, to induce the biologic changes observed. In addition to the varying spleen size and weight, our studies indicate that the enhancement of Ag-specific CTL activity was present only when both p35 and p40 chains were coadministered with the DNA immunogens such as pCGag/pol (Fig. 9*B*). These results indicate that inoculation of both chains are required to induce the biologic effect and the enhancement of cellular immune response in vivo.

Discussion

The generation of immune responses in vivo using DNA inoculation was reported by different laboratories using different therapeutic targets and delivery techniques. We have shown that a nucleic acid delivery approach produced anti-HIV-1 cellular and humoral immune responses in mice as well as in nonhuman primates (6, 7). Ulmer et al. reported that in vivo DNA inoculation using plasmid vectors encoding the influenza A nucleoprotein gene resulted in the production of both humoral and cellular responses as well as evidence of protection from mouse-adapted influenza challenge (4). Similar studies reporting on the use of gene gun technology for delivering nucleic acids on gold bead particles directly into animals using the influenza model Ag system were also reported (20). Following these initial reports, direct DNA inoculation using a variety of in vivo transfection methods into animals have been shown to generate host immunity against a variety of pathogenic Ags such as bovine hepatitis virus, human hepatitis B surface Ag, HTLV-I, hepatitis C viral Ags, malarial Ags, and HIV-1 in a variety of animal systems including mice, rats, rabbits, cows, and nonhuman primates including rhesus macaques and chimpanzees (1–7).

Induction of cell-mediated immunity may be an important feature for any candidate vaccine for HIV. During natural infection, anti-HIV-1 CTL responses appear very early and temporarily appear to correlate with the establishment of the viral set point (32, 33). CTLs play a critical role in viral clearance by targeting and destroying virus-infected cells (34). Directing immune responses against viral proteins through the development of specific CTL responses would allow induction of a more broad immune response against multiple antigenic targets within the virus. The CTL activity against the virus is more readily measured in healthy infected patients as compared with AIDS patients, and specific CTLs have been reported to decrease as disease pathogenesis increases, clearly linking CTL responses with preferred clinical status (35, 36). In this regard, we have reported that a cynomologous macaque with high specific CTL and low Ab responses was protected against a chimeric SHIV challenge, while the animals with low CTL and high Ab responses limited viral replication but were not completely protected (7). Specific CTL responses appear to contribute to the maintenance of the asymptomatic phase of HIV-1 infection. Thus, the induction of strong HIV-1-specific CTLs in vivo through DNA immunization may play a crucial role in the ultimate protection of the host from the progression of HIV infection.

We investigated the potential enhancement of immune responses, especially the CTL response, from DNA vaccines for HIV-1 via codelivery IL-12 and GM-CSF genes as genetic adjuvants. We cloned the genes for IL-12 and GM-CSF into expression vector and injected them into mice along with DNA vaccine cassettes for HIV-1. We have found that coimmunization of plasmids encoding for IL-12 with DNA vaccine for HIV-1 resulted in a dramatic increase in Ag specific CTL response. GM-CSF codelivery appeared to increase humoral responses while IL-12 codelivery suppressed humoral response by about 20%.

Although to our knowledge this is the first reporting on the in vivo use of IL-12 genes as genetic adjuvants for DNA vaccines, several laboratories have reported the effects of delivering cytokine genes to elicit in vivo (37) or in vitro immune responses. Xiang and Ertl have shown that intramuscular coinoculation of plasmid expressing the glycoprotein of rabies virus and plasmids encoding mouse GM-CSF enhanced the B and Th cell activity to rabies virus (28). On the other hand, coinoculation with a plasmid expressing IFN-γ resulted in a decrease of the immune response to rabies virus. Sun et al. have shown that gene gun injection of human IL-6 and TNF-α directly into the tumor cells resulted in reduction of tumor (38). In addition, treatment with murine IL-2 and IFN-γ genes prolonged the survival of Renca tumor-bearing mice and resulted in tumor eradication in 25% of the test animals. Irvine et al. have shown that immunization with IL-2, IL-6, IL-7, and IL-12 genes following DNA inoculation murine tumor CT26 resulted in significant reduction of established lung metastases, while the DNA vaccine immunization alone or treatment with other cytokines (GM-CSF, IFN-γ, and IL-4) did not result in tumor regression (39). In addition, Rakhmlevich et al. found that direct particle-mediated in vivo delivery of IL-12 genes into epidermal cells with implanted intradermal tumor resulted in the regression of established tumor cells (40).

IL-12 may be a particularly important as IL-12 has been reported to be an important inducer and enhancer of Th1-type response and cytotoxic activities. IL-12 is heterodimeric cytokine (p35 and p40 chains) produced by macrophages and B cells. IL-12 induces production of IFN-γ and plays a role in the enhancement of both NK and $CD8^+$ T lymphocyte cytotoxic activities (18, 40). IL-12 treatment of purified NK cells has been reported to enhance their cytotoxic activity in vitro (18). In addition to its role in NK cell cytotoxic activity, treatment of $CD3^+$ peripheral blood T cells with IL-12 resulted in their ability to lyse anti-CD3 Ab-coated FcR-positive targets (40). Numerous in vitro experiments have also shown that IL-12 enhances the generation of allospecific CTL activity by mouse and human $CD8^+$ T lymphocytes (17–19). In addition, we also studied the immunologic effects of the coadministration of GM-CSF genes.

From our experiments, we found many significant immunologic effects of codelivering cytokine genetic adjuvants with DNA vaccines for HIV-1. First, we observed that size and weight of spleens from mice injected with DNA vaccine and IL-12 genes weighed almost three times as much as the control spleens. In addition, the number of white blood cells from these spleens were more than three times the number of cells from the control spleens. These results agree with previous findings that in vivo administration of rIL-12 in mice caused splenomegaly (41–43). Car et al. found that

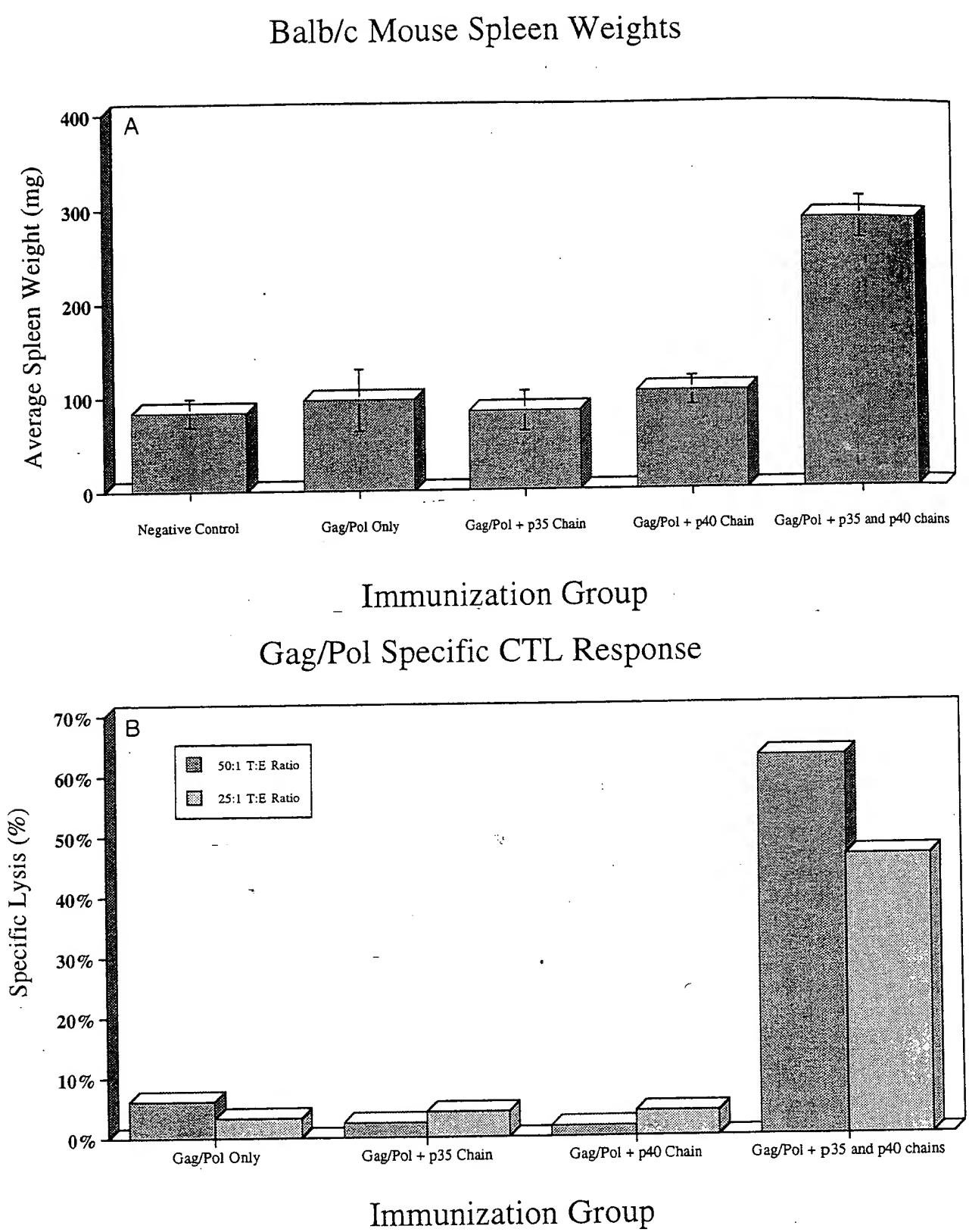

FIGURE 9. *A*, Since p35 chain of IL-12 is thought to be constitutively expressed in many cell types, it may be possible that only a single p40 chain and pCGag/pol were responsible for the biologic and immunologic effects observed in these experiments. To test this, we coadministered 50 μg of each of the two IL-12 heterodimer genes (p35 and p40) with 50 μg of pCGag/pol. At 14 days after immunization spleens were harvested from all immunized animals and were weighed. When either of the two chains were immunized with pCGag/pol, the enlargement of spleen size was not observed. These data indicate that the coinjection with DNA vaccine and both p35 and p40 IL-12 genes resulted in the increased size of spleen and corresponding augmentation of the number of splenic cells, supporting the fact that the plasmids probably entered the same cells in vivo and coordinated dual transcription. *B*, In addition to the varying spleen size and weight, our studies indicate that the enhancement of Ag-specific CTL activity was present only when both p35 and p40 chains were coadministered with the DNA immunogens such as pCGag/pol. These results indicate that inoculation of both p35 and p40 IL-12 chains are required along with DNA immunogen to induce the specific enhancement of cellular immune response in vivo.

a microgram injection of rIL-12 protein resulted in a fivefold increase of spleen weight in wild-type mice (43). These IL-12-induced changes in wild-type mice were associated with markedly increased IFN-γ serum levels. However, IL-12 administration also induced a qualitatively similar (to two times normal) increase in spleen size in IFN-γ receptor-deficient mice (42). These earlier studies reported splenomegaly following injection of IL-12 protein. In this report, we present the utility of delivering a small amount of IL-12 genes in vivo to induce splenomegaly to the level comparable to those published works with rIL-12 proteins. It has been reported that in vivo injection of rIL-12 into mice could have a degree of toxic effects on injected mice such as weight reduction and even death (44–46). It is important to note that our coadministration of IL-12 genes induced enlarged spleens without any visible adverse changes in the injected mice for up to 6 mo after immunization. This suggests that the likely natural processing and sustained low level delivery through plasmid inoculation may be clinically relevant. More importantly, we demonstrate here the power of DNA delivery strategy in inducing significant systemic immune responses without apparent toxicity.

Aside from the induction of splenomegaly, we found that we could direct the specific immune response toward the enhancement of the cellular immune response via the coadministration of IL-12 genes with DNA vaccines. In this regard, we observed that the codelivery of IL-12 genes with DNA vaccine resulted in the reduction of specific Ab response, while the coinjection of GM-CSF genes resulted in the enhancement of specific Ab response. These results agree with the earlier reporting that IL-12 is a key cytokine in directing the immune response from Th2 to Th1 type response. These Ab results were also in agreement with the spleen cell FACS data in which the reduction in the percentage of $B220^+$ B cells was observed with the mice immunized with immunogen (HIV-1 envelop or Gag/Pol) and IL-12 genes. In addition, we also observed a significant Ag-specific stimulation of Th cells with codelivery of IL-12 and GM-CSF with pCGag/pol. The Ag-specific proliferation is a good indicator of $CD4^+$ Th cell immunity, which appears to be a feature of both cytokines.

To further elucidate the T cell response to the DNA coimmunization, we conducted CTL assays both with and without in vitro stimulation of the harvested splenocytes. An enhancement of CTL response is a key evidence in demonstrating the ability to direct immune responses resulting from DNA immunogens from the Th2- to Th1-type response.

We previously reported on the induction of specific CTL response using DNA vaccine constructs with in vitro stimulation CTL assay, but this is the first report of a significant and specific CTL induction by DNA vaccines for HIV-1 against vaccinia-infected targets using an unstimulated CTL assay. In this work, we have found both consistent and dramatic increases in the CTL immune responses by coimmunizing the host with IL-12 genes and various HIV-1 DNA immunogens, pCEnv, pCGag/pol, pCVif, and pCNef. In all of these experiments, we found a significant increase in Ag-specific CTL activity. The enhancement of the Ag-specific CTL response resulted in nearly doubling of the specific lysis of target cells. In addition, we found that the IL-12 coimmunized effectors tested against irrelevant targets did not result in a significant lysis of these targets. These data indicate that the increase in the cytotoxic response following IL-12 coimmunization was due to specific response by T lymphocytes and was not due to NK cells. Furthermore, we conducted CTL assay with a nef peptide (peptide 73–82), which is both MHC class I restricted and $CD8^+$ dependent (29–31). Thus, the results indicate the dramatic increase in CTL was both MHC class I restricted and $CD8^+$ dependent.

The initial goal of the first studies in the field of DNA immunization was to demonstrate the DNA vaccines' ability to elicit humoral and cellular responses in vivo. As we explore the next generation of DNA vaccines, our goal is to refine the current DNA vaccination strategy to elicit more clinically efficacious immune responses. We theorized that such refinement could be accomplished by codelivering genes for immunologically important molecules to help direct and manipulate the type and direction of immune responses, for example to direct responses from Th2 to Th1 type. Specifically in HIV-1 vaccine development, there is data suggesting that the cellular response may be critically important in potentiating the host immune system to prevent viral infection as well as to clear virally infected cells. We chose to investigate the codelivery of cytokine genes with DNA immunogens because cytokines play a critical regulatory and signaling role in immunity. In this study, we specifically chose IL-12 because of the known role IL-12 plays in the induction of Th1-type immune response. By coadministering IL-12 genes with DNA immunogens, we were in fact able to moderately suppress humoral response and dramatically increase the CTL response. In addition we were able to induce splenomegaly, which is a characteristic trait in the in vivo administration of recombinant proteins IL-12 in mice. Thus, we demonstrate the power of DNA delivery in vivo for both the production of a new generation of more effective vaccines and directed immunotherapy as well as an analytic tool for the molecular dissection of the mechanisms of immune function.

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References

1. Wang, B., E. K. Ugen, V. Srikantan, M. Agadjanyan, K. Dang, Y. Refaeli, A. I. Sato, J. D. Boyer, W. V. Williams, and D. B. Weiner. 1993. Gene inoculation generates immune responses against human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA 90:4156.*
2. Wang, B., J. D. Boyer, V. Srikantan, L. Coney, R. Carrano, C. Phan, M. Merva, K. Dang, M. Agadjanyan, L. Gilbert, K. Ugen, V. W. Williams, and D. B. Weiner. 1993. DNA inoculation induces neutralizing immune responses against human immunodeficiency virus type 1 in mice and nonhuman primates. *DNA Cell Biol. 12:799.*
3. Tang, D., M. DeVitand, and S. Johnston. 1992. Genetic immunization is a simple method for elicting an immune response. *Nature 356:152.*
4. Ulmer, J. B., J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. L. Dwarki, S. H. Gromkowski, R. Deck, C. M. DeVitt, A. Friedman, L. A. Hawe, K. R. Leander, D. Marinez, H. Perry, J. W. Shiver, D. Montgomery, and M. A. Liu. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science 259:1745.*
5. Fynan, E., R. Webster, D. Fuller, J. Haynes, J. Santoro, and H. Robinson. 1993. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proc. Natl. Acad. Sci. USA 90:11478.*
6. Wang, B., J. D. Boyer, K. Ugen, V. Srikantan, V. Ayyavoo, M. Agadjanyan, W. Williams, M. Newman, L. Coney, R. Carrano, and D. B. Weiner. 1995. Nucleic acid-based immunization against HIV-1: induction of protective in vivo immune responses. *AIDS 9(Suppl. A):S159.*
7. Boyer, J. D., B. Wang, K. E. Ugen, M. G. Agadjanyan, A. Javadian, P. Frost, K. Dang, R. Carrano, R. Ciccarelli, L. Coney, W. V. Williams, L. Coney, and D. B. Weiner. Protective anti-HIV immune responses in non-human primates through DNA immunization. *J. Med. Primatol. 25:242.*
8. McMichael, A. J., and B. D. Walker. 1994. Cytotoxic T lymphocyte epitopes: implications for HIV vaccines. *AIDS 8(Suppl. 1):S155.*
9. Salk, J., P. A. Bretscher, P. L. Salk, M. Clerici, and G. M. Shearer. 1993. A strategy for prophylactic vaccination against HIV. *Science 260:1270.*
10. O'Sullivan, S. T., J. A. Lederer, A. F. Horgan, D. H. Chin, J. A. Mannick, and M. L. Rodrick. 1995. Major injury leads to predominance of the T helper-2 lymphocyte phenotype and diminished interleukin-12 production associated with decreased resistance to infection. *Ann. Surg. 222:482.*
11. Milich. D. R., S. F. Wolf, J. L. Hughes, and J. E. Jones. 1995. Interleukin 12 suppresses autoantibody production by reversing helper T-cell phenotype in hepatitis B e antigen transgenic mice. *Proc. Natl. Acad. Sci. USA 92:6847.*

12. Druet, P., R. Sheela, and L. Pelletier. 1995. Th1 and Th2 cells in autoimmunity. *Clin. Exp. Immunol. 101(Suppl.1):9.*
13. Liblau, R. S., S. M. Singer, and H. O. McDevitt. 1995. Th1 and Th2 CD4$^+$ T cells in the pathogenesis of organ-specific autoimmune diseases. *Immunol. Today 16:34.*
14. Hetzel, C., G. F. Hoyne, and J. R. Lamb. 1995. Peptide-mediated immunoregulation. *Int. Arch. Allergy Appl. Immunol. 107:275.*
15. Ying, S., S. R. Durham, C. J. Corrigan, Q. Hamid, and A. B. Kay. 1995. Phenotype of cells expressing mRNA for TH2-type (interleukin 4 and interleukin 5) and TH1-type (interleukin 2 and interferon gamma) cytokines in bronchoalveolar lavage and bronchial biopsies from atopic asthmatic and normal control subjects. *Am. J. Respir. Cell Mol. Biol. 12:477.*
16. Kobayashi, M., L. Fitz, M. Ryan, R. M. Hewick, S. C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri. 1989. Identification and purification of natural killer cell stimulator factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med. 10:827.*
17. Gately, M. K., A. G. Wolitzky, P. M. Quinn, and R. Chizzonite. 1992. Regulation of human cytolytic lymphocyte responses by interleukin-12. *Cell. Immunol. 143:127.*
18. Robertson, M. J., R. J. Soiffer, S. F. Wolf, T. J. Manley, C. Donahue, D. Young, S. H. Herrmann, and J. Ritz. 1992. Responses of human natural killer (NK) cells to NK cell stimulatory factor (NKSF): cytolytic activity and proliferation of NK cells are differentially regulated by NKSF. *J. Exp. Med. 175:413.*
19. Bloom, E. T., and J. A. Horvath. 1994. Cellular and molecular mechanisms of the IL-12-induced increase in allospecific murine cytolytic T cell activity: implications for the age-related decline in CTL. *J. Immunol. 152:4242.*
20. Moore, M. A. S. 1991. The clinical use of colony stimulating factors. *Annu. Rev. Immunol. 9:159.*
21. Nicola, N. A. 1991. Hemopoietic cell growth factors and their receptors. *Annu. Rev. Biochem. 58:45.*
22. Costello, R. T. 1993. Therapeutic use of granulocyte-macrophage colony stimulating factor (GM-CSF). *Acta Oncol. 32:403.*
23. Freund, M., and H. D. Kleine. 1992. The role of GM-CSF in infection. *Infection 20:S84.*
24. Takahashi, H., Y. Nakagawa, C. Pendleton, R. Houghten, K. Yokomuro, R. Germain, and J. Berzofsky. 1992. Induction of broadly cross-reactive cytotoxic T cells recognizing an HIV-1 envelope determinant. *Science 255:333.*
25. Shirai, M., C. Pendleton, and J. Berzofsky. 1992. Broad recognition of cytotoxic T cell epitopes from the HIV-1 envelope protein with multiple class I histocompatibility molecules. *J. Immunol. 148:1657.*
26. Trinchieri, G. 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate persistance and antigen-specific adaptive immunity. *Annu. Rev. Immunol. 13:251.*
27. Dranoff, G., E. Jaffee, A. Lazenby, P. Golumbek, H. Levitsky, K. Brose, V. Jackson, H. Hamada, D. Pardoll, and R. C. Mulligan. 1993. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. USA 90:3539.*
28. Xiang, Z., and H. C. Ertl. 1995. Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines. *Immunity 2:129.*
29. Couillin, I., F. Connan, B. Culmann-Penciolelli, E. Gomard, J. Guillet, and J. Choppin. 1995. HLA-dependent variations in human immunodeficiency virus nef protein alter peptide/HLA binding. *Eur. J. Immunol. 25:728.*
30. Couillin, I., F. Connan, B. Culmann-Penciolelli, E. Gomard, J. Choppin, J. Levy, J. Guillet, and S. Saragosti. 1994. Impaired cytotoxic T lymphocyte recognition due to genetic variations in the main immunogenic region of the human immunodeficiency virus 1 nef protein. *J. Exp. Med. 180:1129.*
31. Culmann, B., E. Gomard, M. Kieny, B. Guy, F. Dreyfus, A. Saimot, D. Serini, D. Sicard, and J. Levy. 1991. Six epitopes reacting with human cytotoxic CD8$^+$ T cells in the central region of the HIV-1 nef protein. *J. Immunol. 146:1560.*
32. Koup, R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J. Virol. 68:4650.*
33. Borrow, P., H. Lewicki, H. Hahn, G. M. Shaw, and M. B. A. Oldstone. 1994. Virus-specific CD8$^+$ cytotoxic T lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J. Virol. 68:6103.*
34. Carmichael, A., X. Jin, P. Sissons, and L. Borysiewicz. 1993. Quantitative analysis of human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocyte (CTL) response at different stages of HIV-1 infection: differential CTL responses to HIV-1 and Epstein-Barr virus in late disease. *J. Exp. Med. 177:249.*
35. Klein, M. R., and C. A. van Baalen. 1995. Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics. *J. Exp. Med. 181:1356.*
36. Rinaldo, C., X.-L. Huang, Z. Fan, M. Ding, L. Beltz, L. A. D. Panicali, G. Mazzara, J. Liebmann, M. Cottrill, and P. Gupta. 1995. High levels of anti-human immunodeficiency virus type 1 (HIV-1) memory cytotoxic T-lymphocyte activity and low viral load are associated with lack of disease in HIV-1-infected long-term nonprogressors. *J. Virol. 69:5838.*
37. Raz, E., A. Watanabe, S. M. Baird, R. A. Eisenberg, T. B. Parr, M. Lotz, T. J. Kipps, and D. A. Carson. 1993. Systemic immunological effects of cytokine genes injected into skeletal muscle. *Proc. Natl. Acad. Sci. 90:4523.*
38. Sun, W. H., J. K. Burkholder, J. Sun, J. Culp, J. Turner, X. G. Lu, T. D. Pugh, W. B. Ershler, and N. S. Yang. 1995. In vivo cytokine gene transfer by gene gun reduces tumor growth in mice. *Proc. Natl. Acad. Sci. USA 92:2889.*
39. Irvine, K. R., J. B. Rao, S. A. Rosenberg, and N. P. Restifo. 1996. Cytokine enhancement of DNA immunization leads to treatment of established pulmonary metastases. *J. Immunol. 156:238.*
40. Rakhmlevich, A., J. Turner, M. Ford, D. McCabe, W. Sun, P. Sondel, K. Grota, and N. Yang. 1996. Gene gun-mediated skin transfection with interleukin 12 gene results in regression of established primary and metastatic murine tumors. *Proc. Natl. Acad. Sci. USA 93:6291.*
41. Bonnema, J. D., K. A. Rivlin, A. T. Ting, R. A. Schoon, R. T. Abraham, and P. J. Leibson. 1994. Cytokine-enhanced NK cell-mediated cytotoxicity: positive modulatory effects IL-2 and IL-12 on stimulus-dependent granule exocytosis. *J. Immunol. 152:2098.*
42. Tare, N. S., S. Bowen, R. R. Warrier, D. M. Carvajal, W. R. Benjamin, J. H. Riley, T. D. Anderson, and M. K. Gately. 1995. Administration of recombinant interleukin-12 to mice suppresses hematopoiesis in the bone marrow but enhances hematopoiesis in the spleen. *J. Interferon Cytokine Res. 15:377.*
43. Car, B. D., V. M. Eng, B. Schnyder, M. LeHir, A. N. Shakhov, G. Woerly, S. Huang, M. Aguet, T. D. Anderson, and B. Ryffel. 1995. Role of interferon-gamma in interleukin 12-induced pathology in mice. *Am. J. Pathol. 147:1693.*
44. Eng, V. M., B. D. Car, B. Schnyder, M. Lorenz, S. Lugli, M. Aguet, T. D. Anderson, B. Ryffel, and V. F. Quesniaux. 1995. The stimulatory effects of interleukin (IL)-12 on hematopoiesis are antagonized by IL-12-induced interferon gamma in vivo. *J. Exp. Med. 181:1893.*
45. Stevenson, M. M., M. F. Tam, S. F. Wolf, and A. Sher. 1995. IL-12-induced protection against blood-stage *Plasmodium chabaudi* AS requires IFN-γ and TNF-α and occurs via a nitric oxide-dependent mechanism. *J. Immunol. 155:2545.*
46. Orange, J. S., T. P. Salazar-Mather, S. M. Opal, R. L. Spencer, A. H. Miller, B. S. McEwen, and C. A. Biron. 1995. Mechanism of interleukin 12-mediated toxicities during experimental viral infections: role of tumor necrosis factor and glucocorticoids. *J. Exp. Med. 181:901.*
47. Gazzinelli, R. T., N. A. Giese, and H. C. Morse III. 1994. In vivo treatment with interleukin 12 protects mice from immune abnormalities observed during murine acquired immunodeficiency syndrome (MAIDS). *J. Exp. Med. 180:2199.*

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Construction of a Single-Chain Interleukin-12-Expressing Retroviral Vector and Its Application in Cytokine Gene Therapy against Experimental Coccidioidomycosis

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T-cell-mediated immunity is an important determinant in protection against primary infection with *Coccidioides immitis*, a dimorphic fungal pathogen that causes the disease coccidioidomycosis. To determine if interleukin-12 (IL-12) gene therapy could potentiate host response against *C. immitis*, we constructed a single-chain cDNA encoding the p40 and p35 subunits linked by a polylinker and, using a retroviral vector, transfected J774 macrophages with the construct. The transduced J774 cells expressed IL-12 in vitro, with a mean concentration of 28,440 pg from 10^6 cells in 48 h as measured by an IL-12 (p75)-specific enzyme-linked immunosorbent assay. The secreted IL-12 was biologically active, as judged by its ability to induce the production of gamma interferon (IFN-γ) by spleen cells from BALB/c mice. Treatment of the highly susceptible BALB/c mouse strain with the IL-12-transduced J774 cells inhibited *C. immitis* growth in tissues from mice challenged by a pulmonary route, as evidenced by 1.37-, 2.59-, and 1.22-log reductions in the number of CFU in the lungs, spleens, and livers, respectively, compared to the fungal load in mice given vector-transduced J774 cells. The protective effect of IL-12 gene therapy was accompanied by increased levels of IFN-γ in the lungs and sera of mice treated with IL-12-transduced J774 cells and the constitutive production of IFN-γ by their spleen cells cultured in vitro. These results suggest that IL-12 gene therapy could be used as adjunct therapy for coccidioidomycosis.

Coccidioidomycosis is a mycotic disease caused by the dimorphic fungus *Coccidioides immitis*. The disease is endemic in the semiarid areas of Texas, Arizona, New Mexico, and southern California. Primary infection is acquired by inhalation of mycelial-phase arthroconidia, which enter the alveoli and undergo a morphologic conversion into endosporulating spherules (35). Coccidioidomycosis presents a diverse clinical spectrum, ranging from benign, self-limited pulmonary infection to a severe, progressive, and often lethal extrapulmonary dissemination. Investigations in humans and experimentally infected animals have shown strong T-cell reactivity to coccidioidal antigens in subjects with controlled infection, whereas T-cell responses are depressed or nondemonstrable in subjects with progressive, multifocal disease (4, 6, 12–15, 26, 27). Recovery from primary asymptomatic or benign infection with *C. immitis* confers lifelong immunity to exogenous reinfection. The acquired resistance is associated with the acquisition of a delayed-type hypersensitivity response and the production of T helper-1 (Th1)-associated cytokines, such as gamma interferon (IFN-γ) and interleukin-2 (IL-2), to coccidioidal antigens (4, 6, 12–15, 26, 27).

IL-12, a heterodimeric Th1-promoting cytokine consisting of two disulfide-bonded subunits of 35 and 40 kDa, has been shown to have potent immunotherapeutic effects against tumor cells and a wide range of microbial pathogens (7, 11, 16, 20, 28, 29, 40, 42, 43). This cytokine has pleiotropic effects, including activation of macrophages, augmentation of the cytolytic activity of NK and T cells, and induction of the Th1-associated cytokines, notably IFN-γ (38). In a previous study, we showed that IL-12 plays a critical role in host defense against *C. immitis* (27). Treatment of the highly susceptible BALB/c mouse strain with recombinant murine IL-12 (mIL-12) ameliorated the course of the disease and enhanced production of IFN-γ. Daily injections of 0.1 μg of the recombinant cytokine were required, however, to achieve optimal therapeutic effects. Since gene therapy offers a means for constitutive production of a protein in vivo, we engineered a retroviral construct containing the cDNA encoding the p40 and p35 subunits of IL-12 as a single chain. Treatment of BALB/c mice with J774 macrophages that had been transduced with the single-chain IL-12 retroviral construct afforded a significant level of protection against lethal pulmonary challenge with *C. immitis* and was accompanied by increased production of IFN-γ.

MATERIALS AND METHODS

Animal model. Pathogen-free female BALB/c mice, 5 to 7 weeks of age, were purchased from Jackson Laboratory (Bar Harbor, Maine) and used throughout this study. These animals arrived in filtered cages and were maintained for 1 more week before use.

Cell lines. The J774A.1 (J774) and PA317 cell lines were obtained from the American Type Culture Collection, Rockville, Md. (ATCC TIB-67 and CRL-9078, respectively). The J774 cell line is a BALB/c-derived reticulum cell sarcoma having macrophage-like properties (30). The PA317 cell line was derived from NIH 3T3 TK fibroblast cells. The cell lines were maintained at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, N.Y.), supplemented with 2 mM glutamine, 100 U of penicillin/ml, 100 μg of streptomycin/ml, 4,500 mg of glucose/ml, and 10% fetal bovine serum (Atlanta Biological, Inc., Norcross, Ga.).

Cloning of single-chain murine IL-12 into retroviral vector. The cDNAs for murine IL-12 p35 and p40 subunits were generously provided by Ueli Gubler (Hoffmann-La Roche, Nutley, N.J.) (32). We introduced an *Xho*I site at the 5′ end of p40 and a *Bgl*II site at the 3′ end of p35 by PCR amplification, using synthetic oligonucleotide primers for p40 and p35, with a polylinker on the 3′ end of the p40 primer and the 5′ end for the p35 primer. The primers contained the following sequences (5′ to 3′): for p40, upstream TCTAGAGGCTCGAGCCC CACCAT and downstream TGGAATGACCCTAGATCCGCCGCCACCCGA CCCACCACCGCCCGAGCCACCGCCACCGGATCGGACCCT; and for p35, upstream AGGGTCCGATCCGGTGGCGGTGGCTCGGGCGGTGGTGGG

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TCGGGTGGCGGCGGATCTAGGGTCATTCCA and downstream CGCTCT AGATCTAGTCGACCAATG. A 35-cycle PCR was performed to amplify the p40 and p35 fragments by high-fidelity PCR (Boehringer, Mannheim, Germany) under the following conditions: 94°C for 5 min; 94°C for 1 min, 55°C for 1.5 min, and 68°C for 1.5 min; and 72°C for 7 min. The single-chain PCR-amplified fragment for mIL-12 was constructed by linkage of the p35 and p40 cDNA fragments with the upstream primer of p40 and the downstream primer of p35 in a second round of PCR (22). The 1.66-kb PCR fragment of mIL-12 was directly cloned into PCR 2.1 vector (TA cloning kit; Invitrogen, San Diego, Calif.) and sequenced to confirm the nucleotide sequence of the adjacent subunit regions, linker, and the p40 and p35 coding regions. Thereafter, the single-chain cDNA of mIL-12 was ligated into the pLXSN vector (Clontech, Palo Alto, Calif.). The pLXSN/p40.LΔp35 construct, hereafter referred to as pLXSN/mIL-12, contains the gene encoding neomycin resistance as a selectable marker. The immunopotentiating capacity of the pLXSN/mIL-12 construct was compared with that of the pLXSN vector alone.

Cell transfection. The pLXSN/mIL-12 construct was transduced into the amphotropic packaging cell line PA317 by calcium phosphate precipitation (Gibco). After a 72-h incubation, supernatants were collected from the transduced cells and assayed for IL-12 by using the p75 enzyme-linked immunosorbent assay (ELISA) procedures as described below. Transfectants were selected by culturing the cells for 2 weeks in complete DMEM containing G418 (Gibco) at a concentration of 800 μg/ml. IL-12 viral particle-producing PA317 clones were identified by reverse transcription (RT)-PCR (for both neomycin resistance gene and IL-12 mRNAs) and by a p75 ELISA as described below. IL-12-producing PA317 clones were expanded in DMEM, and virus-containing supernatant was harvested and used to infect J774 macrophages.

Expression of bioactive IL-12 by transduced J774 cells. To test the ability of the pLXSN/mIL-12 constructs to induce the expression of mIL-12, total RNA was isolated from 5×10^6 transduced J774 cells and assayed for IL-12 mRNA by RT-PCR. PCR amplification was performed with primer pairs for β-actin (Clontech), the neomycin resistance gene (41), and IL-12 (p40Δp35 as described above) mRNA transcripts. Expression of IL-12 at the protein level was determined by a sandwich ELISA devised specifically to detect IL-12 p75. The capture antibody was a hamster immunoglobulin G (IgG) anti-mIL-12 monoclonal antibody (Red-T; PharMingen, San Diego, Calif.), which reacts with mIL-12 p35 and the p75 heterodimer but not the p40 monomer. Captured IL-12 was detected by the addition of biotinylated rat IgG2a anti-mIL-12 p40 (clone C17.8). Recombinant mIL-12 (PharMingen) was used to prepare a standard curve.

The bioactivity of the secreted IL-12 was assayed by measuring its ability to induce IFN-γ production by spleen cells from nonimmune mice. For these experiments, spleens were collected from normal BALB/c mice and gently teased into single-cell suspensions. The spleen cell suspension was treated with isotonic ammonium chloride to lyse erythrocytes and, after being washed by centrifugation, the splenocytes were resuspended in DMEM containing 10% fetal bovine serum. The cells were dispensed into wells on a microtiter plate at a concentration of 2×10^6 mononuclear cells per well. The cell cultures were incubated in medium alone or in medium containing a 1:10 dilution of the supernatant from the IL-12-transduced J774 cells. After a 48-h incubation at 37°C under 5% CO_2, supernatants were collected for assays of IFN-γ protein by a two-site sandwich ELISA by using rat IgG1 anti-mouse IFN-γ monoclonal antibodies from clones R4-6A2 for capture and biotinylated XMG1.2 for detection (PharMingen) as previously reported (19). Recombinant mouse IFN-γ (PharMingen) was used to establish a standard curve.

Infection of mice. The procedure for infecting mice via a pulmonary route has been detailed in an earlier report (13). In brief, arthroconidia were harvested from 6- to 8-week-old mycelial-phase cultures of *C. immitis* Silveira (ATCC 28868). The arthroconidial suspension was passed over a nylon column to remove hyphal elements, and the cells were enumerated by hemacytometer counts. Pulmonary challenge was performed by intranasal instillation of 60 arthroconidia in 30 μl of physiologic saline.

Gene therapy. J774 cells, transduced with pLXSN/mIL-12 or with pLXSN alone, were administered via an intraperitoneal (i.p.) route 6 h after pulmonary challenge and again on days 1, 4, and 7. Control mice were treated in the same manner with saline alone. Mice were sacrificed at day 12 after challenge, and the lungs, livers, and spleens were collected, weighed, and homogenized. Serial dilutions of the homogenates were plated on mycobiotic medium (Difco Laboratories, Detroit, Mich.) for enumeration of fungal CFU.

To assess the effect of IL-12 gene therapy on the Th1 response in vivo, mice treated with the pLXSN/mIL-12-transduced J774 cells, vector-transduced J774 cells, or saline alone were sacrificed at 12 days postinfection, and their serum, lungs, and spleens were collected for assays of IFN-γ. Prior to assay, the lungs were suspended in sterile saline, homogenized in sterile Whirl-Pak bags (American Scientific Products, Dallas, Tex.), and filtered through a 22-μm-pore-size membrane. The filtered lung homogenates and the serum samples were maintained at −70°C until assayed by the ELISA described above. For assays of IFN-γ production by spleen cells, splenocytes (2×10^6) were incubated in tissue culture medium alone or medium containing concanavalin A (ConA, 2 μg; Sigma Chemical Co., St. Louis, Mo.). Forty-eight hours later, the spleen cell supernatants were collected and assayed for IFN-γ by ELISA.

Statistical analysis. The statistical significance of differential findings between experimental groups of animals was determined by the nonparametric Mann-Whitney rank sum test. Findings were regarded as significant if two-tailed *P* values were <0.05.

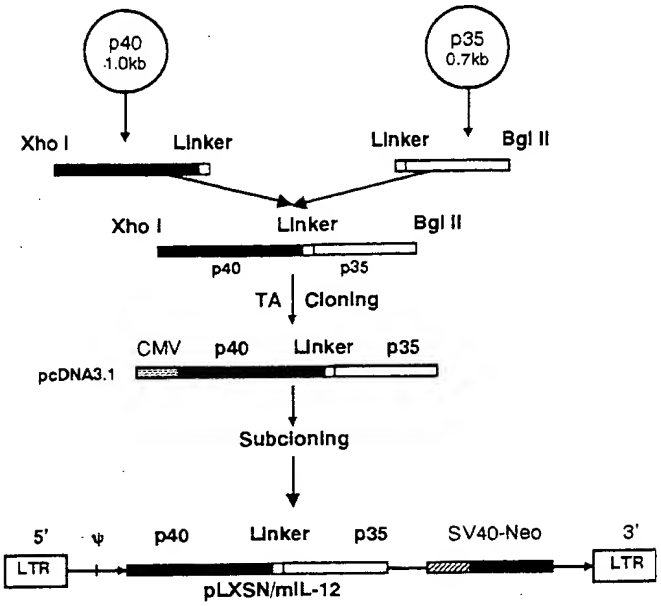

FIG. 1. Schematic representation of the retroviral construct that carries both the p40 and p35 genes and the neomycin resistance gene selectable marker. The p40 and p35 cDNAs were linked by a $(Gly_4Ser)_3$ polylinker and were regulated by the long terminal repeat (LTR) promoter. This plasmid vector, designated pLXSN/mIL-12, was capable of coordinately expressing the p40, p35, and neomycin resistance genes. CMV, cytomegalovirus; SV40, simian virus 40.

RESULTS

Expression of bioactive mIL-12 in transduced cells. Generation of the functionally active IL-12 heterodimer requires the expression of both the p40 and the p35 genes (21, 22, 32). Although this can be achieved by simultaneously transfecting cells with two separate plasmids encoding the p40 and p35 genes, excessive p40 expression has been shown to lead to the inhibition of the bioactivities of IL-12 in mice (9, 17). To avoid this potential problem, we generated a single-chain construct containing both the p40 and the p35 genes (32), connected by a 45-bp linker encoding 15 amino acids (22), with the neomycin resistance gene as a selectable marker. This polycistronic construct is depicted in Fig. 1.

J774 cells were infected by coculture with viral particle-containing supernatant from pLXSN/mIL-12-transduced PA317 cells or PA317 cells that had been transduced with the pLXSN vector alone. The transduced J774 cell clones were obtained after a 2-week selection with G418 and examined for expression of IL-12 at both the molecular and protein levels by RT-PCR and IL-12 ELISA, respectively. The results obtained by the RT-PCR assays are shown in Fig. 2. J774 cells transduced with pLXSN/mIL-12 expressed mRNAs for both full-length 1.66-kb IL-12 and the 0.68-kb neomycin resistance gene, while vector-transduced J774 cells expressed mRNA only for the neomycin resistance gene. IL-12 was also detected when the supernatants from transduced J774 cells were assayed by ELISA, with a mean level of 28,440 pg from 10^6 cells in 48 h (Fig. 3A). The secreted IL-12 was bioactive, as evidenced by the induction of 3,300 pg of IFN-γ in resting spleen cells (Fig. 3B). These IL-12-transduced J774 cells have constitutively produced bioactive IL-12 for over 1 year and have retained their original morphology, growth pattern, and expression of the cell

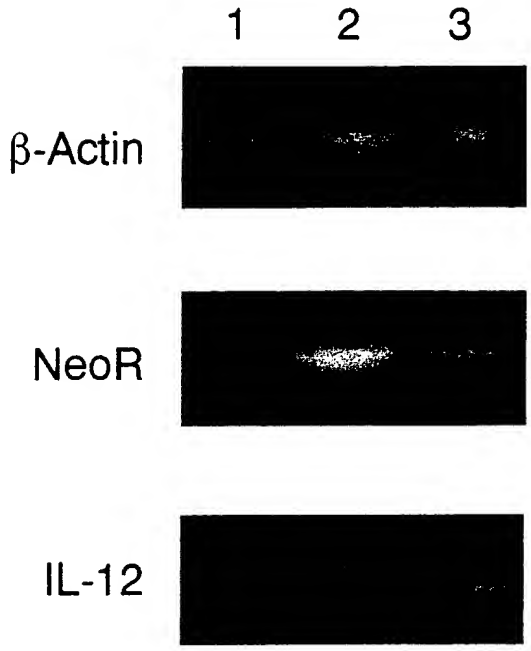

FIG. 2. Expression of mRNA transcripts for β-actin, the neomycin resistance gene, and mIL-12 in pLXSN/mIL-12-J774 cells. Lanes 1, 2, and 3 depict the results obtained with cellular RNA obtained from nontransduced J774 cells, J774 cells transduced with the pLXSN plasmid vector alone, and J774 cells transduced with the pLXSN/mIL-12 construct, respectively.

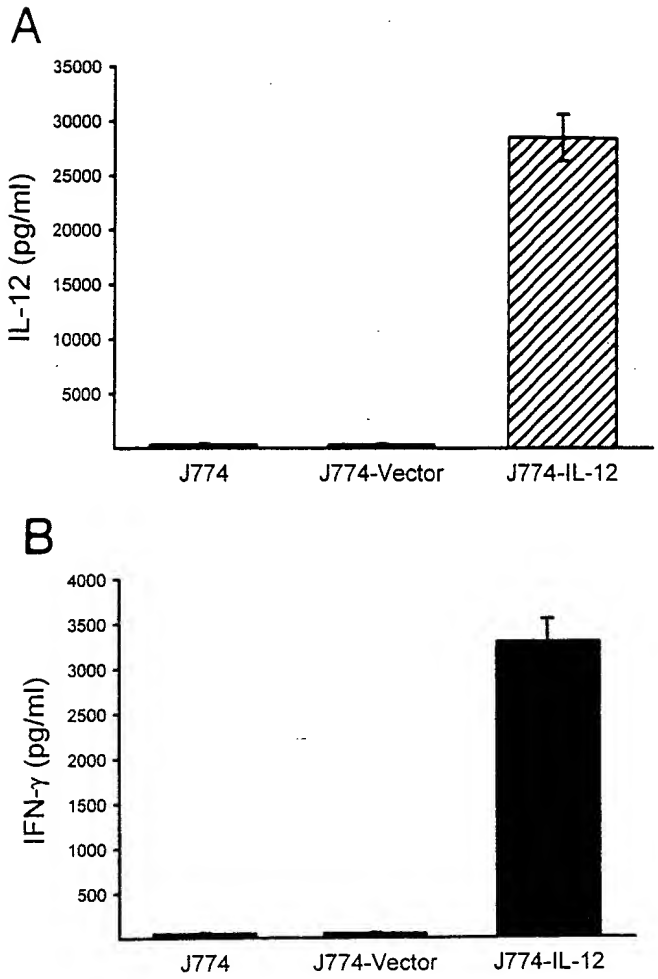

FIG. 3. Secretion of bioactive IL-12 from pLXSN/mIL-12-transduced J774 cells. Supernatants were collected at 48 h from in vitro cultures of 10^6 pLXSN/mIL-12-transduced J774 cells and, for negative controls, nontransduced and vector-transduced J774 cells. The supernatants were assayed for IL-12 by ELISA (A) and for bioactive IL-12 (B) as measured by the induction of IFN-γ production in (2×10^6) spleen cells from normal BALB/c mice. Results are representative of those obtained in at least two separate experiments.

surface markers Mac-3, major histocompatibility complex class I (MHC-I), and MHC-II, as measured by flow cytometry (data not shown).

Treatment of mice with IL-12-transduced J774 cells protects against pulmonary challenge with *C. immitis*. To examine the capacity of IL-12-transduced J774 cells to protect mice against challenge, BALB/c mice were infected with 60 arthroconidia via a pulmonary route and then treated with 2×10^6 pLXSN/mIL-12-transduced J774 cells or vector-transduced J774 cells. Twelve days after challenge, the mice were sacrificed and examined for fungal CFU. The results, depicted in Fig. 4, established that mice treated with pLXSN/mIL-12-transduced J774 macrophages showed a significant decrease in the number of *C. immitis* CFU in the lungs compared to the vector ($P < 0.001$) and saline control groups ($P < 0.001$). Recipients of the IL-12-transduced J774 cells also showed significant reductions in the fungal load in their spleens ($P < 0.0001$) and livers ($P < 0.001$).

It is perhaps noteworthy that mice given pLXSN/mIL-12-transduced J774 cells, but not those receiving vector-transduced cells, showed marked splenomegaly, with a greater-than-twofold increase in weight compared to spleens from mice treated with the vector-transduced J774 cells. This finding is consistent with a recent report by Kim et al. (21) that mice given an IL-12 cDNA expression vector showed a level of splenomegaly that was comparable to that observed in mice given recombinant IL-12.

In vivo induction of Th1 response in mice given IL-12 gene therapy. The preceding results established that retroviral vector-mediated IL-12 gene therapy effected a significant decrease in the fungal load in the lungs, livers, and spleens of mice. To determine if this protection was accompanied by induction of IFN-γ, mice treated with the IL-12-transduced J774 or nontransduced cells were sacrificed 12 days after challenge, and their sera and lung homogenates were assayed for IFN-γ. As shown in Table 1, sera from mice treated with the pLXSN/mIL-12-transduced J774 cells showed 1,580 pg of IFN-γ per ml, whereas no IFN-γ was detected in sera from mice treated with the vector-transduced J774 cells or saline. Likewise, the mean IFN-γ level in lung homogenates from mice treated with the pLXSN/mIL-12-transduced J774 cells was 1,300 pg/100 mg of tissue, compared to 200 pg and <15 pg in lung tissue from mice treated with the pLXSN vector or saline alone, respectively. Further proof that the transduced J774 cells induced IFN-γ production in vivo was evidenced by our finding that spleen cells from mice treated with the pLXSN/mIL-12-transduced J774 cells secreted 290 pg of IFN-γ when cultured in vitro in medium alone (Table 2). No IFN-γ was detected when splenocytes from mice given saline alone or vector-transduced J774 cells were incubated in medium alone. When the spleen cells were assayed for IFN-γ production in response to stimulation with ConA, cells from recipients of IL-12-transduced J774 cells secreted 3,900 pg compared to 60 and 160 pg by splenocytes from recipients of saline alone or vector-transduced J774 cells, respectively. The decreased production of IFN-γ by ConA-stimulated spleen cells from the latter two

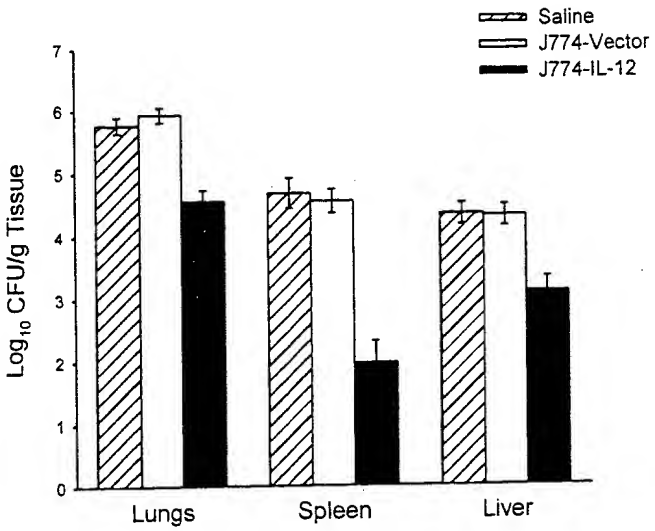

FIG. 4. Protection in BALB/c mice treated with pLXSN/mIL-12-transfected J774 cells. Mice were infected with 60 arthroconidia via a pulmonary route and then treated with 2×10^6 IL-12-transduced or vector-transduced J774 cells via the i.p. route. A third group of mice received saline alone. Treatments were begun 6 h after pulmonary challenge and repeated on days 1, 4, and 7 after challenge. Twelve days postchallenge, the mice were sacrificed and evaluated for *C. immitis* CFU in tissues. Bars depict means ± standard errors obtained in two experiments involving a total of 22 mice given saline alone, 23 mice treated with vector-transduced J774 cells, and 21 mice given IL-12-transduced J774 cells.

groups of mice is consistent with the immunosuppressive effect of active coccidioidomycosis on Th1-associated responses (12, 13, 15, 26).

DISCUSSION

The results of this investigation demonstrate the efficacy of IL-12 gene therapy with a genetically engineered single-chain IL-12 fusion construct created by linkage of the p35 and p40 genes with a $(Gly_4Ser)_3$ polylinker. The monomeric nature of this single-chain IL-12 fusion protein ensures equimolar expression of each subunit, thereby avoiding the formation of p40 dimers which have been shown to antagonize the activity of IL-12 (9, 17). The bioactivity and efficacy of gene therapy with this construct were examined by treating BALB/c mice, which are highly susceptible to *C. immitis* (13), with J774 cells transduced with the single-chain IL-12 construct. Recipients of the IL-12-transduced J774 cells showed a reduced fungal load in their lungs, livers, and spleens after pulmonary challenge and an increased production of the Th1-associated cytokine IFN-γ.

It is now clearly established that IL-12 plays a pivotal role in orchestrating the immune response by amplifying cytokine networks involved in the induction of Th1 cells while suppressing

TABLE 1. IFN-γ levels in sera and homogenates of lung tissues from infected mice

Source	IFN-γ level (pg) for indicated treatment group[a]		
	Saline	J774-Vector	J774-IL-12
Serum	<15[b]	<15	1,580
Lung homogenates	<15	200	1,300

[a] IFN-γ levels per milliliter of serum and per 100 mg of lung tissue obtained from groups of 10 mice at 12 days postinfection.
[b] Lower limit of sensitivity of the assay.

TABLE 2. Production of IFN-γ in vitro by spleen cells from infected mice

Stimulant	IFN-γ secretion (pg) by splenocytes from BALB/c mice treated with[a]:		
	Saline	J774-Vector	J774-IL-12
Medium	<15[b]	<15	290
ConA (2 μg)	60	160	3,900

[a] IFN-γ levels per milliliter of supernatant from 2×10^6 spleen cells from a pool of at least 11 mice at 12 days postinfection.
[b] Lower limit of assay sensitivity.

Th2 responses (38). In murine studies, recombinant IL-12 has been shown to prevent growth of a wide spectrum of tumors (7, 22, 29) and to augment host resistance to several pathogens, including *Mycobacterium tuberculosis* (11), *Listeria monocytogenes* (40), *Toxoplasma gondii* (20), *Leishmania major* (28), *Schistosoma mansoni* (42), *Histoplasma capsulatum* (43), *Cryptococcus neoformans* (16), and *C. immitis* (27). The limitations of recombinant cytokine therapy include the need for daily administrations, often with significant systemic toxicity (27, 45), and the inability to target the cytokine to a specific organ or tissue site. To address these limitations, investigators have focused on using gene transfer therapy for the in vivo production of bioactive IL-12. This approach has proved to be highly effective as evaluated in experimental tumor models (8, 9, 17, 21–23, 30, 36, 45) and infectious diseases (1, 10, 18, 37, 39, 44) and appears to be without toxicity.

We have previously reported that IL-12 has an essential role in host defense against *C. immitis* (27). Administration of 0.1 μg of recombinant IL-12 to susceptible BALB/c mice on the day before pulmonary challenge with *C. immitis* and then daily for 12 days afterward resulted in a significant reduction in the fungal load in the spleens and livers but not the lungs. Protection at the lung level was not achieved even with the administration of a 10-fold-higher dose of the recombinant protein. These results and the finding that mice treated with the higher dose showed toxic manifestations, evidenced by ruffled fur, lethargy, and a marked reduction in total body weight by 8 days postinfection (27), led us to examine the efficacy of IL-12 gene therapy. In this investigation, we used a single-chain IL-12 retroviral construct expressed in J774 cells to provide a potent and stable delivery system for bioactive IL-12. Treatment of BALB/c mice with the IL-12-expressing J774 cells effected a reduction in the fungal load in tissues and induced IFN-γ production, as evidenced by increased levels of IFN-γ in serum and lungs from treated mice 12 days after challenge with *C. immitis*. The induction of IFN-γ is an important consequence of IL-12 gene therapy, since this cytokine has been shown to activate macrophages to an anticoccidioidal level, both in vitro and in vivo (5, 14).

The protective effect of IL-12 gene therapy has in other models been shown to be attributable to the induction of the IFN-γ by NK cells and T lymphocytes and to the subsequent development of Th1 responses (10, 11, 16, 36, 38–40, 42, 43). The in vivo elaboration of IFN-γ in the mice given IL-12-transduced J774 cells is consistent with a role of IL-12 in the activation of antifungal host defense via the induction of this Th1-associated cytokine. It is also possible that the IL-12-transduced J774 macrophages themselves were activated to an anticoccidioidal level via in situ expression of the IL-12 gene. We chose to use the J774 cell line for delivery of the pLXSN/mIL-12 retroviral construct because investigators have shown that J774 cells are highly effective for expressing retroviral

vectors containing mycobacterial genes (25, 33, 34). Although we did not initially consider that IL-12 transduction of the J774 cells might enhance their antimicrobial activity, this possibility should be explored by comparing the anticoccidioidal effect of IL-12-transduced J774 cells with that of IL-12-transduced cells of a nonmacrophage lineage. Studies should also be done to examine the therapeutic efficacy of IL-12-transduced dendritic cells, since dendritic cells have been reported to be highly effective for expressing cytokine genes in the therapy of cancer and infectious diseases (2, 3, 24, 31).

Cytokine gene therapy is a promising approach for inducing efficient immune responses against infectious diseases. We have demonstrated the feasibility of IL-12 gene therapy for the treatment of coccidioidomycosis by retrovirally-transduced J774 cells. To our knowledge, this study is the first to show that gene therapy with a single-chain IL-12 fusion construct will induce protective immunity and increase IFN-γ production in a fungal disease. The results are extremely encouraging and indicate that IL-12 gene therapy has potential as adjunct therapy for coccidioidomycosis.

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REFERENCES

1. **Ahlers, J. D., N. Dunlop, D. W. Alling, P. L. Nara, and J. A. Berzofsky.** 1997. Cytokine-in-adjuvant steering of the immune response phenotype to HIV-1 vaccine constructs. Granulocyte-macrophage colony-stimulating factor and TNF-α synergize with IL-12 to enhance induction of cytotoxic T lymphocytes. J. Immunol. **158**:3947–3958.
2. **Ahuja, S. S., M. R. Brown, T. A. Fleisher, S. K. Ahuja, and H. L. Malech.** 1996. Autocrine activation of hemopoietic progenitor-derived myelo-monocytic cells by IFN-γ gene transfer. J. Immunol. **156**:4345–4353.
3. **Ahuja, S. S., S. Mummidi, H. L. Malech, and S. K. Ahuja.** 1998. Human dendritic cells (CD)-based anti-infective therapy: engineering dendritic cells to secrete functional IFN-γ and IL-12. J. Immunol. **161**:868–876.
4. **Ampel, N. M., G. C. Bejarano, S. D. Salas, and J. N. Galgiani.** 1992. In vitro assessment of cellular immunity in human coccidioidomycosis: relationship between dermal hypersensitivity, lymphocyte transformation, and lymphokine production by peripheral blood mononuclear cells from healthy adults. J. Infect. Dis. **165**:710–715.
5. **Beaman, L.** 1987. Fungicidal activation of murine macrophages by recombinant gamma interferon. Infect. Immun. **55**:2951–2955.
6. **Beaman, L., D. Pappagianis, and E. Benjamini.** 1977. Significance of T cells in resistance to experimental murine coccidioidomycosis. Infect. Immun. **17**:580–585.
7. **Brunda, M. J., L. Luistro, R. R. Warrier, R. B. Wright, B. R. Hubbard, M. Murphy, S. F. Wolf, and M. K. Gately.** 1993. Antitumor and antimetastatic activity of interleukin 12 against murine tumors. J. Exp. Med. **178**:1223–1230.
8. **Car, B. D., V. M. Eng, B. Schnyder, M. LeHir, A. N. Shakhov, G. Woerly, S. Huang, M. Aguet, T. D. Anderson, and B. Ryffel.** 1995. Role of interferon-γ in interleukin 12-induced pathology in mice. Am. J. Pathol. **147**:1693–1707.
9. **Chen, L., D. Chen, E. Block, M. O'Donnell, D. W. Kufe, and S. K. Clinton.** 1997. Eradication of murine bladder carcinoma by intratumor injection of a bicistronic adenoviral vector carrying cDNAs for the IL-12 heterodimer and its inhibition by the IL-12 p40 subunit homodimer. J. Immunol. **159**:351–359.
10. **Chow, Y.-H., B.-L. Chiang, Y.-L. Lee, W.-K. Chi, W.-C. Lin, Y.-T. Cheu, and M.-H. Tao.** 1998. Development of Th1 and Th2 populations and the nature of the immune responses to hepatitis B virus DNA vaccines can be modulated by codelivery of various cytokine genes. J. Immunol. **160**:1320–1329.
11. **Cooper, A. M., A. D. Roberts, E. R. Rhoades, J. E. Callahan, D. M. Getzy, and I. M. Orme.** 1995. The role of interleukin-12 in acquired immunity to *Mycobacterium tuberculosis*. Immunology **84**:423–432.
12. **Corry, D. B., N. M. Ampel, L. Christian, R. M. Locksley, and J. N. Galgiani.** 1996. Cytokine production by peripheral blood mononuclear cells in human coccidioidomycosis. J. Infect. Dis. **174**:440–443.
13. **Cox, R. A., W. Kennell, L. Boncyk, and J. W. Murphy.** 1988. Induction and expression of cell-mediated immune responses in inbred mice infected with *Coccidioides immitis*. Infect. Immun. **56**:13–17.
14. **Cox, R. A., and D. M. Magee.** 1998. Protective immunity in coccidioidomycosis. Res. Immunol. **149**:417–428.
15. **Cox, R. A., and J. R. Vivas.** 1977. Spectrum of in vivo and in vitro immune responses in coccidioidomycosis. Cell. Immunol. **31**:130–141.
16. **Decken, K., G. Kohler, K. Palmer-Lehmann, A. Wunderlin, F. Mattner, J. Magram, M. K. Gately, and G. Alber.** 1998. Interleukin-12 is essential for a protective Th1 response in mice infected with *Cryptococcus neoformans*. Infect. Immun. **55**:4994–5000.
17. **Gillessen, S., D. Carvajal, P. Ling, F. J. Podlaski, D. L. Stremlo, P. C. Familletti, U. Gubler, D. H. Presky, A. S. Stern, and M. K. Gately.** 1995. Mouse interleukin-12 (IL-12) p40 homodimer: a potent IL-12 antagonist. Eur. J. Immunol. **23**:200–206.
18. **Greenberger, M. J., S. Kunkel, R. M. Strieter, N. W. Lukacs, J. Bramson, J. Gauldie, F. L. Graham, M. Hitt, J. M. Danforth, and T. J. Standiford.** 1996. IL-12 gene therapy protects mice in lethal *Klebsiella pneumoniae*. J. Immunol. **157**:3006–3012.
19. **Jiang, C., D. M. Magee, T. N. Quitugua, and R. A. Cox.** 1999. Genetic vaccination against *Coccidioides immitis*: comparison of vaccine efficacy of recombinant antigen 2 and antigen 2 cDNA. Infect. Immun. **67**:630–635.
20. **Johnson, L. L., and P. C. Sayles.** 1997. Interleukin-12, dendritic cells, and the initiation of host-protective mechanisms against *Toxoplasma gondii*. J. Exp. Med. **186**:1799–1802.
21. **Kim, J. J., V. Ayyavoo, M. L. Bagarazzi, M. A. Chaattergoon, K. Dang, B. Wang, J. D. Boyer, and D. B. Weiner.** 1997. In vivo engineering of a cellular immune response by coadministration of IL-12 expression vector with a DNA immunogen. J. Immunol. **158**:816–826.
22. **Lieschke, G. J., P. K. Rao, M. K. Gately, and R. C. Mulligan.** 1997. Bioactive murine and human interleukin-12 fusion proteins which retain antitumor activity in vivo. Nat. Biotechnol. **15**:35–40.
23. **Lotze, M. T., L. Zitvogel, R. Campbell, P. D. Robbins, E. Elder, C. Haluszczak, D. Martin, T. L. Whiteside, W. J. Storkus, and H. Tahara.** 1996. Cytokine gene therapy of cancer using interleukin-12: murine and clinical trials. Ann. N. Y. Acad. Sci. **795**:440–454.
24. **Lotze, M. T., B. Hellerstedt, L. Stolinski, T. Tueting, C. Wilson, D. Kinzler, H. Vu, J. T. Rubin, W. Storkus, H. Tahara, E. Elder, and T. Whiteside.** 1993. The role of interleukin-2, interleukin-12, and dendritic cells in cancer therapy. Cancer J. Sci. Am. **3**(Suppl.):S109–S114.
25. **Lowrie, D. B., R. E. Tascon, M. J. Colston, and C. L. Silva.** 1994. Towards a DNA vaccine against tuberculosis. Vaccine **12**:1537–1540.
26. **Magee, D. M., and R. A. Cox.** 1995. Roles of gamma interferon and interleukin-4 in genetically determined resistance to *Coccidioides immitis*. Infect. Immun. **63**:3514–3519.
27. **Magee, D. M., and R. A. Cox.** 1996. Interleukin-12 regulation of host defenses against *Coccidioides immitis*. Infect. Immun. **64**:3609–3613.
28. **Murray, H. W., and J. Harisprashad.** 1995. Interleukin-12 is effective treatment for an established systemic intracellular infection: experimental visceral leishmaniasis. J. Exp. Med. **181**:387–391.
29. **Nastala, C. L., H. D. Edington, T. G. McKinney, H. Tahara, M. A. Nalesnik, M. J. Brunda, M. K. Gately, S. F. Wolf, R. D. Schreiber, and W. J. Storkus.** 1994. Recombinant IL-12 administration induces tumor regression in association with IFN-γ. J. Immunol. **153**:1697–1705.
30. **Ralph, P., J. Prichard, and M. Cohn.** 1975. Reticulum cell sarcoma: an effector cell in antibody-dependent cell-mediated immunity. J. Immunol. **114**:898–904.
31. **Reid, C. D.** 1998. The biology and clinical applications of dendritic cells. Transfusion Med. **8**:77–86.
32. **Schoenhaut, D. S., A. O. Chua, A. G. Wolitzky, M. Phyllis, P. M. Quinn, C. M. Dwyer, W. McComas, P. C. Familletti, M. K. Gately, and U. Gubler.** 1992. Cloning and expression of murine IL-12. J. Immunol. **148**:3433–3440.
33. **Silva, C. L., and D. B. Lowrie.** 1994. A single mycobacterial protein (hsp 65) expressed by a transgenic antigen-presenting cell vaccinates mice against tuberculosis. Immunology **82**:244–248.
34. **Silva, C. L., A. Palacios, M. J. Colston, and D. B. Lowrie.** 1992. *Mycobacterium leprae* 65 hsp antigen expressed from a retroviral vector in a macrophage cell line is presented to T cells in association with MHC class II in addition to MHC class I. Microb. Pathog. **12**:27–38.
35. **Stevens, D. A.** 1995. Current concepts: coccidioidomycosis. N. Engl. J. Med. **332**:1077–1082.
36. **Tahara, H., L. Zitvogel, W. J. Storkus, H. J. Zeh III, T. G. McKinney, R. D. Schreiber, U. Gubler, P. D. Robbins, and M. T. Lotze.** 1995. Effective eradication of established murine tumors with IL-12 gene therapy using a polycistronic retroviral vector. J. Immunol. **154**:6466–6474.
37. **Tascon, R. E., M. J. Colston, S. Ragno, E. Stavropoulos, D. Gregory, and D. B. Lowrie.** 1996. Vaccination against tuberculosis by DNA injection. Nat. Med. **2**:888–898.
38. **Trinchieri, G.** 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu. Rev. Immunol. **152**:1883–1887.
39. **Tsuji, T., K. Hamajima, J. Fukushima, K. Q. Xin, N. Ishii, I. Aoki, Y. Ishigatsubo, K. Tani, S. Kawamoto, Y. Nitta, J. Miyazaki, W. C. Koff, T. Okubo, and K. Okuda.** 1997. Enhancement of cell-mediated immunity against HIV-1 induced by coinoculation of plasmid-encoded HIV-1 antigen with plasmid expressing IL-12. J. Immunol. **158**:4008–4013.

40. **Wagner, R. D., H. Steinberg, J. F. Brown, and C. J. Czuprynski.** 1994. Recombinant interleukin 12 enhances resistance to mice to *Listeria monocytogenes* infection. Microb. Pathog. 17:175–186.
41. **Westermann, J., A. Aicher, Z. Qin, Z. Cayeux, K. Daemen, T. Blankenstein, B. Dorken, and A. Pezzutto.** 1998. Retroviral interleukin-7 gene transfer into human dendritic cells enhances T cell activation. Gene Ther. 5:264–271.
42. **Wynn, T. A., D. Jankovic, S. Hieny, A. W. Cheever, and A. Sher.** 1995. IL-12 enhances vaccine-induced immunity to *Schistosoma mansoni* in mice and decreases T helper 2 cytokine expression, IgE production, and tissue eosinophilia. J. Immunol. 154:4701–4709.
43. **Zhou, P., M. C. Sieve, J. Bennett, K. J. Kwon-Chung, R. P. Tewari, R. T. Gazzinelli, A. Sher, and R. A. Seder.** 1995. IL-12 prevents mortality in mice infected with *Histoplasma capsulatum* through induction of IFN-γ. J. Immunol. 155:785–795.
44. **Zhu, Z., N. Venkataprasad, H. S. Thangaraj, M. Hill, M. Singh, J. Ivanyi, and H. M. Vordermeier.** 1997. Functions and specificity of T cells following nucleic acid vaccination of mice against *Mycobacterium tuberculosis* infection. J. Immunol. 158:5921–5926.
45. **Zitvogel, L., H. Tahara, P. D. Robbins, W. J. Storkes, M. R. Clarke, M. A. Nalesnik, and M. T. Lotze.** 1995. Cancer immunotherapy of established tumors with IL-12. Effective delivery by genetically engineered fibroblasts. J. Immunol. 155:1393–1403.

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Intradermal Delivery of IL-12 Naked DNA Induces Systemic NK Cell Activation and Th1 Response In Vivo That Is Independent of Endogenous IL-12 Production[1]

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In this study four murine IL-12 naked DNA expression plasmids (pIL-12), containing both the p35 and p40 subunits, were shown to induce systemic biological effects in vivo after intradermal injection. Three of the four IL-12 expression vectors augmented NK activity and induced expression of the IFN-γ and IFN-γ-inducible Mig genes. Both IL-12 p70 heterodimer and IFN-γ proteins were documented in the serum within 24 h after intradermal injection of the pIL-12o$^-$ plasmid, which also induced the highest level of NK activity in the spleen and liver among the IL-12 constructs. Interestingly, both p40 mRNA expression at the injection site and serum protein levels followed a biphasic pattern of expression, with peaks on days 1 and 5. Subsequent studies revealed that the ability of intradermally injected pIL-12o$^-$ to augment NK lytic activity was prevented by administration of a neutralizing anti-IL-12 mAb. Finally, injection of the pIL-12o$^-$ into BALB/c IL-12 p40$^{-/-}$ mice also resulted in a biphasic pattern of IL-12 p70 appearance in the serum, and induced IFN-γ protein and activated NK lytic activity in liver and spleen. These results demonstrate that injection of delivered naked DNA encoding the IL-12 gene mediates the biphasic systemic production of IL-12-inducible genes and augments the cytotoxic function of NK cells in lymphoid and parenchymal organs as a direct result of transgene expression. The results also suggest that these naked DNA plasmids may be useful adjuvants for vaccines against infectious and neoplastic diseases. *The Journal of Immunology,* **1999, 163: 1943–1950.**

IL-12 was initially identified and isolated as an NK cell stimulatory factor (1). Compared with other cytokines, it has a unique 70-kDa heterodimeric structure composed of two covalently linked p35 and p40 subunits, both of which are required for biological activities (1, 2). IL-12 is produced principally by APC, such as monocytes, macrophages, and dendritic cells. In addition to this stimulatory effect on NK cells, IL-12 activates cytotoxic T cells (3–5), differentiates CD4$^+$ lymphocytes (6, 7), plays an important role in regulating the balance between the type 1 and type 2 response of Th lymphocytes (8, 9), primes macrophages for nitric oxide production (10), and possesses IFN-γ-dependent antiangiogenic activity (11, 12). These diverse biological effects make IL-12 an attractive candidate as a therapeutic agent for cancer and infectious diseases. Systemic administration of IL-12 protein alone (2, 13–15) or in combination with IL-2 (16–18) significantly suppressed the growth of a variety of established mouse tumors and prolonged the survival of tumor-bearing mice. IL-12 also has efficacy as an adjuvant for vaccination against cancer (19), and intratumoral delivery of adeno- or retroviruses containing the IL-12 gene can cause regression of some established tumors in mice (20–23). The targeted inactivation of both alleles of the IL-12 p40 gene impairs the production of IFN-γ and the induction of a delayed-type hypersensitivity response (24) and renders mice susceptible to infection by *Leishmania major* (24).

Despite these interesting therapeutic implications for IL-12, the best approaches for delivery of IL-12 in vivo remain to be determined. Most studies have been performed using systemic delivery of the rIL-12 protein. Although the pharmacodynamics of IL-12 are more favorable than those of many other cytokines, repeated administration on a daily basis is required for maximal therapeutic activity in mice (25, 26). In addition, the repeated bolus administration of the recombinant proteins can cause undesirable side effects (27, 28). Alternative approaches for IL-12 delivery also have some limitations. For example, virus-mediated gene delivery can result in the subsequent generation of neutralizing Ab, which limits the duration that active immunotherapy is effective. In the case of cytokine-mediated retroviruses, integration of the virus genome into host chromosomes may be a concern for other deleterious effects.

The direct in vivo transfer of DNA without any carrier agents (referred to as naked DNA) was first described in 1990 as a novel form of gene therapy (29). The initial studies showed that muscle was a suitable target tissue for gene delivery (29, 30), but skin also was shown to be suitable as an alternative site for injection (31–34). Some initial success of naked DNA encoding therapeutic

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proteins was documented by the induction of a host immune response against several infectious agents (31, 35, 36). Subsequently, naked DNA was also proven to induce local or systemic biological effects in vivo, including improvement of anemia by in vivo delivery of the erythropoietin gene (37) or recruitment of neutrophils into the site of IL-8 plasmid DNA injection (32). Major advantages for the in vivo use of highly pure plasmids include the relatively simple and inexpensive production compared with protein and the possibility that more chronic production may decrease the need for high systemic protein levels associated with bolus administration of cytokines, thereby reducing unfavorable side effects.

In this study we constructed several IL-12 expression vectors that encode both murine p35 and p40. These plasmids were injected intradermally (i.d.)[4] and shown to induce IL-12 mediated biological activity, including activation of NK cells, induction of IFN-γ, and the IFN-γ-inducible chemokine monokine induced by IFN-γ (MIG). These effects also were detectable in IL-12 $p40^{-/-}$ mice, showing that they were not mediated by endogenous production of normal host IL-12 or contaminating endotoxin. Overall, these results show that in vivo transferred pIL-12 DNA can induce the expected systemic bioactivities of IL-12 and suggest that this form of gene therapy is efficient and safe for IL-12 delivery.

Materials and Methods

Animals

Pathogen-free female BALB/c mice between 6–8 wk of age were obtained from the Animal Production Area, National Cancer Institute-Frederick Cancer Research and Development Center. These mice were housed under specific pathogen-free conditions and provided sterilized mouse chow and water ad libitum. BALB/c IL-12 $p40^{-/-}$ mice (38) were donated by Dr. Jean Magram, Hoffmann-La Roche (Nutley, NJ) and maintained as a small breeding colony in our own animal facility. Animal care was provided in accordance with the procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell lines and reagents

The hybridoma C17.8 for anti-mouse IL-12 p70 was a gift from Dr. Giorgio Trinchieri (Wistar Institute of Anatomy and Biology, Philadelphia, PA). YAC-1 and P815 cells were maintained in vitro in RPMI 1640 containing 10% FCS and 2 mM glutamine.

Plasmids

The expression vector CMV-β, which encodes the β-galactosidase gene, was obtained from Clontech (Palo Alto, CA). Four constructs of murine pIL-12 were generated as follows. First, the pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA) was modified by inserting an SV40 intron between the CMV enhancer/promoter and multicloning site. As shown in Fig. 1, the p35 and p40 subunits of murine IL-12 were each driven by a separate CMV promoter, and individual expression cassettes were oriented in either the same or opposite directions in a single retroviral vector, except in the case of the pIL-12 IRES plasmid, where the p40 sequence was driven by the IRES. For some plasmids, the neomycin expression cassette was removed. Plasmids were prepared using the Qiagen Endofree Buffer kit and Qiagen-tip 2500 (Qiagen, Valencia, CA) and dissolved in PBS. The endotoxin levels of the prepared plasmids were <0.05 EU/μg of DNA by limulus amoebocyte lystate test (BioWhittaker, Walkersville, MD).

In vivo delivery of naked DNA

Fifty micrograms of plasmid DNA in 100 μl of PBS was injected i.d. at the base of the tail of the mice using a 30-gauge needle and a 1-ml syringe. For i.v. delivery, DNA was dissolved in PBS containing 5% glucose in a total volume of 200 μl and injected via the lateral tail veins.

β-Galactosidase staining

After i.d. injection of CMV-β as described above, the site of gene transfer was harvested at 24 h and fixed in PBS containing 2% formaldehyde and 0.2% glutaraldehyde. The skin was then stained with Bluo-gal (Life Technologies, Gaithersburg, MD) as described previously (32). Briefly, tissue was rinsed in PBS and incubated in PBS containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, and 1 mg/ml Bluo-gal (Life Technologies) at room temperature overnight. On the next day, the tissue was rinsed with PBS and postfixed in 4% formaldehyde, 100 mM sodium phosphate, and 10% methanol.

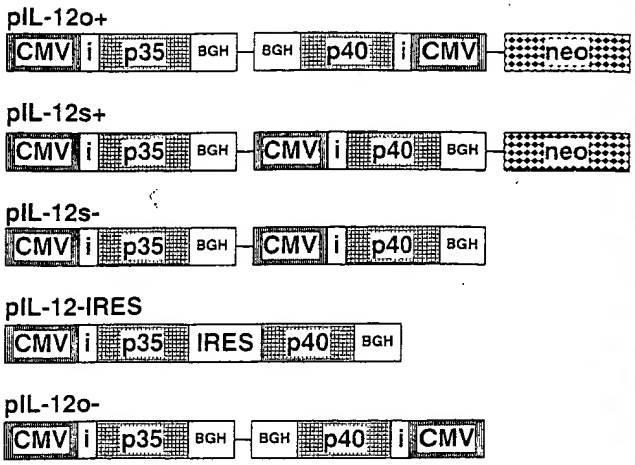

FIGURE 1. Schematic structure of murine IL-12 expression vectors. CMV, CMV enhancer and promoter; i, SV40 intron; p35, mouse IL-12 p35 subunit; p40, mouse IL-12 p40 subunit; BGH, bovine growth hormone polyadenylation site; neo, SV40 early promoter and origin followed by neomycin resistance gene and polyadenylation signal. All the plasmids contain the same backbone from pcDNA3.1$^+$, which includes the replication origin and ampicillin resistance gene.

Detection and quantification of murine IL-12 and IFN-γ by ELISA

Blood was collected at various times from mice after injection of plasmid DNA, and the serum was assayed for murine IL-12 and IFN-γ by ELISA kits purchased from Endogen (Woburn, MA) and R&D Systems (Minneapolis, MN), respectively.

Assessment of NK cell activity in leukocytes isolated from liver and spleen

At various times after plasmid injection, mice were euthanized, and blood, liver, and spleen were harvested. The livers were perfused with HBSS, and mononuclear cells were prepared as previously described (18). Briefly, three or four livers were dissociated on a stomacher (Tekmar, Cincinnati, OH) and centrifuged at 500 × *g*. The resuspended pellet was filtered with nylon gauze, overlaid on Lympholyte M (Ceder Lane Laboratories, Ontario, Canada), and centrifuged at 2600 × *g* for 30 min. The leukocyte layer was recovered, and the cells were washed and counted. Various numbers of leukocytes were then cocultured for 4 h with 1×10^{4} ^{51}Cr-labeled YAC-1 or P815 target cells in 96-well microplates.

RT-PCR

Total RNA was prepared from snap-frozen spleens using Trizol (Life Technologies). cDNA were synthesized with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) primed with an oligo$(dT)_{12-18}$ primer (Pharmacia, Piscataway, NJ) in the presence of 0.2 mM dNTP and 10 U of RNase inhibitor (Pharmacia). cDNA (250 ng) was used to amplify the IFN-γ and Mig genes. The reaction was performed as 30 cycles at 94°C for 30 s, 55°C for 60 s, and 72°C for 60 s. The sequences for primers are: IFN-γ sense, 5′-TGCGGCCTAGCTCTGAGACAATGA 3′; IFN-γ antisense, 5′-TGAATGCTTGGCGCTGGACCTGTG-3′; Mig sense, 5′-GATCAAACCTGCCTAGATCC-3′; Mig antisense, 5′-GGCT GTGTAGAACACAGAGT-3′; actin sense, 5′-CAGCTGAGAGGGAA ATCGTG-3′; and actin antisense, 5′-ACTGTGTTGGCATAGAGGTC-3′. Ten microliters of PCR product was resolved in a 1.5% agarose gel along with a 100-bp m.w. marker (Life Technologies).

Northern blot analysis

Total RNA was prepared at various times as described above from the skin at the site where DNA was injected. Five micrograms of total RNA was run

[4] Abbreviations used in this paper: i.d., intradermal(ly); MIG, monokine induced by IFN-γ; IRES, internal ribosomal entry site.

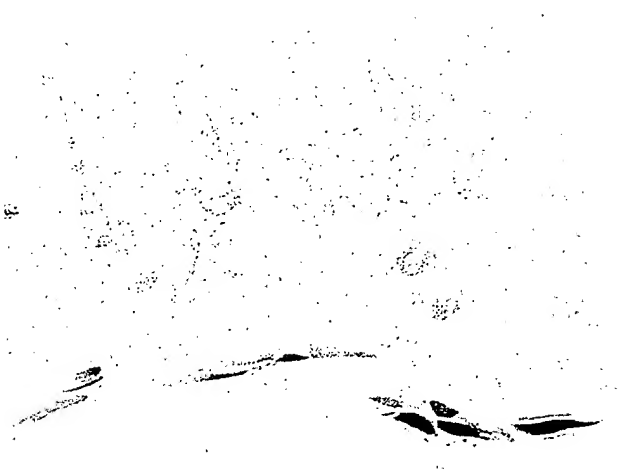

FIGURE 2. In situ expression of the β-galactosidase gene. Thirty micrograms of CMV-β in 30 μl of PBS was injected i.d. at the tail base of BALB/c mice. Twenty-four hours later the skin was harvested and stained with Bluo-gal as described in *Materials and Methods*. β-Galactosidase was detected mainly in smooth muscle in the dermis.

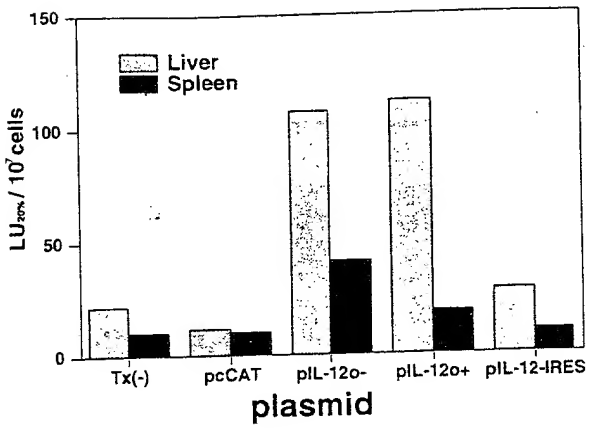

FIGURE 3. Comparison of pIL-12o$^-$, pIL-12$^+$, and pIL-12 IRES for the ability to augment NK activity. Fifty micrograms of each construct was injected once i.d. Four days later the liver and spleen were harvested, and mononuclear cells prepared from these organs were incubated with ^{51}Cr-labeled YAC-1 cells for 4 h.

in 1% agarose gels containing 1× MOPS buffer and transferred to a nylon membrane Hybond N (Amersham, Arlington Heights, IL). The RNA blot was prehybridized in Easy Hyb (Boehringer Mannheim, Indianapolis, IN) and hybridized overnight in the same buffer containing 100 ng/ml of digoxygenin-labeled murine IL-12 p40 antisense riboprobe. The membrane was then washed twice with 0.1% SDS and 0.1× SSC at 65°C for 15 min, and chemiluminescence was detected with CSPD as a substrate according to the manufacturer's handbook (The DIG System User's Guide for Filter Hybridization, Boehringer Mannheim).

Results

Intradermal gene expression

To confirm that i.d. injected DNA actually leads to expressed genes in vivo, we initially investigated the expression of the i.d. injected β-galactosidase gene, CMV-β, as a model system. Twenty-four hours after the i.d. injection of 20 μg of CMV-β, β-galactosidase activity was readily demonstrated at the site of injection (data not shown). Histological analysis of the site revealed the activity was mainly in the smooth muscle of the s.c. tissue (Fig. 2).

Systemic augmentation of NK activity by various IL-12 gene-encoding plasmids

These studies were performed to compare the abilities of the various IL-12 plasmids to augment mouse NK activity in vitro. In preliminary screening studies, leukocytes were isolated from liver and spleen 4 days after the i.d. injection of control or IL-12 expression vectors, and NK-mediated lytic activity was measured against YAC-1 target cells. In these studies pIL-12o$^+$, in which the p35 and p40 expression cassettes were placed in opposite orientations in a single retroviral vector (Fig. 1) gave the highest NK cell activity in the liver and spleen, followed by pIL-12 IRES, pIL-12s$^-$, and pIL-12s$^+$ in the order of NK activity induced (data not shown). The results suggested that the use of separate expression cassettes for p40 and p35 produced higher NK cell activity than that obtained employing the IRES strategy, particularly when the p40 and p35 expression cassettes were placed in opposite orientations. The results also suggested that the presence of the neomycin expression cassette may have some inhibitory effect on IL-12 production and NK activity in vivo. Therefore we constructed the pIL-12o$^-$ in which the p35 and p40 genes were oriented in the opposite direction from each other and where both were under separate control of CMV promoters, but the neomycin gene expression cassette was deleted. The study shown in Fig. 3 compares the NK-augmenting effects of the neo$^-$ and neo$^+$ constructs. NK lytic activity induced by pIL-12o$^-$ was comparable to that obtained with IL-12o$^+$ and was 4- and 3-fold higher than levels induced by the IRES vector in the liver and spleen, respectively. Because there was no apparent benefit to expressing neomycin at the site of gene delivery, the IL-12o$^-$ plasmid was selected for further study to exclude any concerns about whether any observed in vivo biological effects could be related to the expression of neomycin in vivo. None of the mice that received single injections of either pcDNA control vector or the pIL-12 constructs showed any gross toxicities.

Induction of IFN-γ and Mig genes in the spleen

Because many of the biological effects of IL-12 are known to be mediated via induction of IFN-γ and subsequent induction of other IFN-γ-inducible genes, we studied the inducibility of the IFN-γ and Mig genes. Portions of the spleens were obtained from mice treated with the pIL-12o$^-$ and/or pIL-12o$^+$ plasmids alone or with pcDNA vector. Twenty-four hours after the i.d. injection of pIL-12o$^-$, modest induction of IFN-γ gene expression was demonstrated in the spleen by RT-PCR (Fig. 4*A*). Mig gene expression also was observed in the spleen (Fig. 4*B*) 4 days after the injection of pIL-12o$^+$ and pIL-12o$^-$.

Kinetics of NK lytic activity and IL-12 production induced by pIL-12o$^-$

The previous data demonstrated that NK cell activity was induced by pIL-12o$^-$ by day 4. However, it was unclear whether this was the optimal time for detecting augmented NK activity and how this related to the production of IL-12. Therefore, a more detailed kinetic evaluation of these events was performed at various times between 1–14 days after i.d. plasmid injection using the same dose of DNA described above. As shown in Fig. 5 under this time scheme, only at 5 days after pIL-12o$^-$ injection was there a clear increase in NK activity in the liver and spleen.

The data in Fig. 6 depict the kinetics of IL-12 and IFN-γ in sera pooled from three mice per group at various times after injection of 50 μg of pIL-12o$^-$ or pcDNA. As expected, the serum levels of these two cytokines peaked well before the detectable augmentation of NK activity (Fig. 5). Specifically, there was detectable IL-12 (Fig. 6*A*) and IFN-γ protein (Fig. 6*B*) by 24 h after DNA

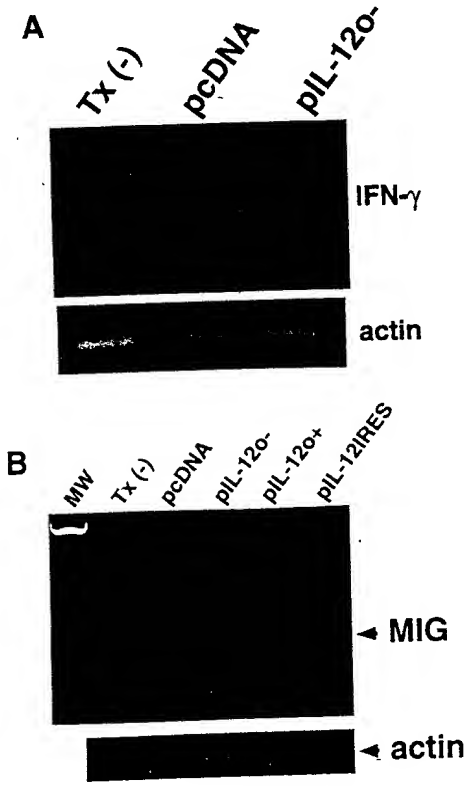

FIGURE 4. *A*, IFN-γ gene expression by RT-PCR. Total RNA was prepared from the spleens of the mice 24 h after the injection of plasmids. *B*, The expression of the Mig gene in the spleen 4 days after the injection of either control or pIL-12.

injection. Surprisingly, the IL-12 protein rose again 96 h after injection, thereby displaying a biphasic profile of IL-12 p70 protein in the serum after the single i.d. injection of pIL-12o⁻.

Expression of p40 gene at the site of DNA injection

To investigate the mechanism for the biphasic production of IL-12 protein, tissue was harvested from the injection site at various times after the injection of pIL-12o⁻ and analyzed by Northern blot for p40 gene expression (Fig. 7). These results showed that by 24 h there was a pronounced induction of the p40 gene, which was then down-regulated by day 3 and re-elevated on day 5, consistent with the protein data presented in Fig. 6*A* above. The gene re-expression initially detected on day 5 remained until day 7. No p40

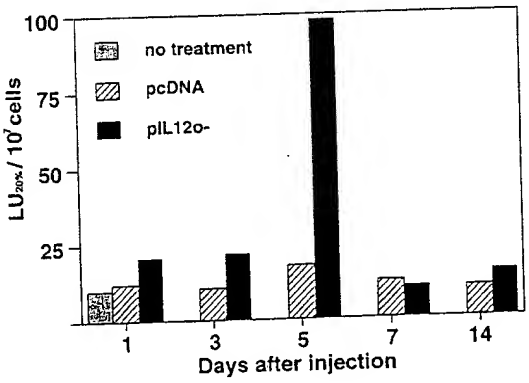

FIGURE 5. Kinetics of NK lytic activity after a single injection of pIL-12o⁻ or pcDNA. Leukocytes were obtained from the livers and spleens of mice at various times after the i.d. injection of DNA. The cells were then cocultured with ^{51}Cr-labeled YAC-1 target cells for 4 h.

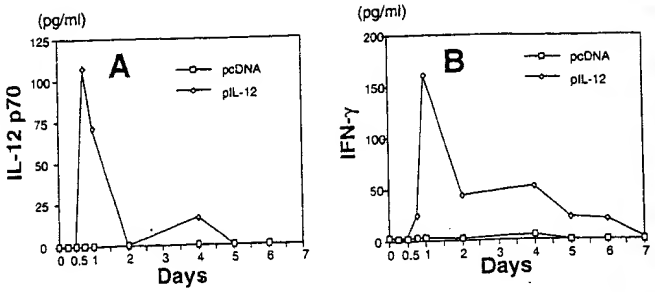

FIGURE 6. The detection and kinetics of serum IL-12 (*A*) and IFN-γ (*B*) proteins after a single injection of pIL-12o⁻ or pcDNA. Fifty micrograms of DNA was injected i.d. The blood from three mice that received the same treatment was combined, and serum was obtained for assay. The results shown are paired samples obtained from the experiment described in Fig. 5.

expression was detected after pcDNA injection, demonstrating that the gene expression observed above was directly due to transcription of the injected pIL-12o⁻. Overall, these results suggest that the biphasic expression of IL-12 protein occurred because of a biphasic expression of the IL-12 gene.

Inhibition of NK activity by anti-IL-12 mAb

To confirm that the systemic NK activity elicited by pIL-12o⁻ was directly dependent on the production of IL-12 protein, mice were pretreated i.p. with 25 μg of either C17.8 rat mAb, which specifically neutralizes the murine IL-12 p70 heterodimer, or control rat Ab beginning 24 h before the injection of 50 μg of pIL-12o⁻ or pcDNA injection. Spleens and livers were then harvested, and isolated leukocytes were tested for NK activity. In this experiment, because of a limited availability of leukocytes from the liver, a single E:T cell ratio was employed to test NK activity, while the assay for splenic NK activity was conducted as in the previous experiment. Mice treated with control Ab and pIL-12o⁻ exhibited the expected increase in NK activity, while those treated with C17.8 Ab and pIL-12o⁻ showed no augmentation of NK activity (Fig. 8).

The i.v. administration of pIL-12o⁻ also results in detectable levels of serum IL-12 and IFN-γ

The data presented above cumulatively support the ability of a locally injected IL-12 expression plasmid to induce systemically

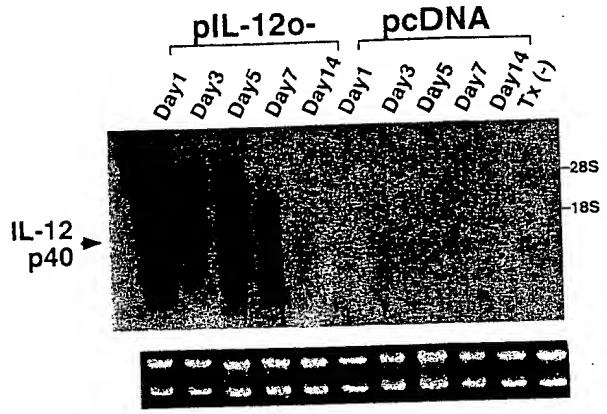

FIGURE 7. Gene expression of the p40 subunit at the site of injection after a single injection of pIL-12o⁻ or control vector. The skin from the site where the DNA was injected was harvested at various time points. Total RNA was hybridized with a digoxigenin-labeled mouse p40 antisense riboprobe. After stringent washing, the membrane was exposed to x-ray film for 1 h.

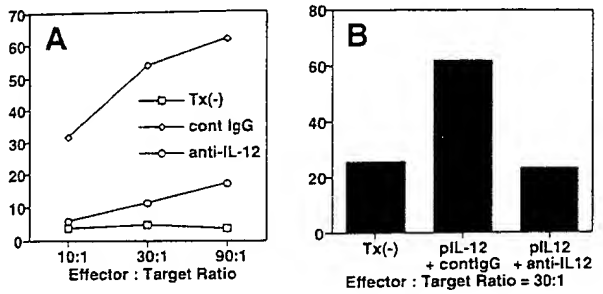

FIGURE 8. Blockade of augmentation of NK lytic activity by pIL-12o$^-$ using Abs against mouse IL-12. Twenty-five micrograms of C17.8 mAb or control IgG was injected i.p. daily starting on the day before the pIL-12o$^-$ injection and continuing until the day before harvest. Fifty micrograms of pIL-12o$^-$ was injected i.d. Four days later leukocytes from the liver (*A*) and spleen (*B*) were harvested, and NK lytic activity was measured in a 4-h ^{51}Cr release assay. Because of a limited availability of leukocytes recovered from the liver, a single E:T cell ratio was employed for this NK lysis assay. Splenic NK activity was assayed as previously described.

detectable cytokine levels and biological effects. However, it is possible that direct localization of the expression plasmid in major organs and the focused production of IL-12 in such sites might have more potent biological effects in specific organs. Therefore, we investigated whether i.v. injected plasmid DNA could augment NK activity in the spleen and liver. This approach resulted in very potent induction of NK activity by 2 days after iv injection of the IL-12o$^-$ plasmid (Fig. 9). This augmented NK activity was retained at 96 h, and the levels of NK activity achieved in the liver were quite high compared with those observed after i.d. injection (Fig. 3).

NK cell activation by pIL-12o$^-$ in p40$^{-/-}$ mice

To completely exclude the possibility that any of the biological effects outlined above were due to the induction of endogenous IL-12 production rather than to direct transcription and translation of the gene product of the injected plasmid, we repeated the NK augmentation studies in IL-12 p40$^{-/-}$ mice. After i.d. injection of 50 μg of pIL-12o$^-$ into the BALB/c p40$^{-/-}$ mice, IL-12 p70 was detected in the serum by 24 h (Fig. 10*A*). IFN-γ also was demonstrated in the serum by 24 h (Fig. 10*B*), and as seen in normal mice, the effect was biphasic, showing high levels at 24 h, a decline, and subsequent re-elevation by 4 days. This IFN-γ induction was confirmed by RT-PCR of spleen from the same treatment mice (Fig. 10*C*). The pIL-12o$^-$ also augmented NK activity in the spleens of the p40$^{-/-}$ mice as previously observed in normal mice (Fig. 11) by day 4. However, in contrast to results in normal mice (Fig. 3), augmentation of NK activity in p40$^{-/-}$ mice occurred more rapidly (by 24 h) and persisted for 4 days. These results demonstrate that the injection of an appropriately constructed pIL-12 naked DNA plasmid can directly contribute enough IL-12 protein to mediate potent systemic production of IL-12-inducible genes and augment the cytotoxic function of NK cells in lymphoid and parenchymal organ sites.

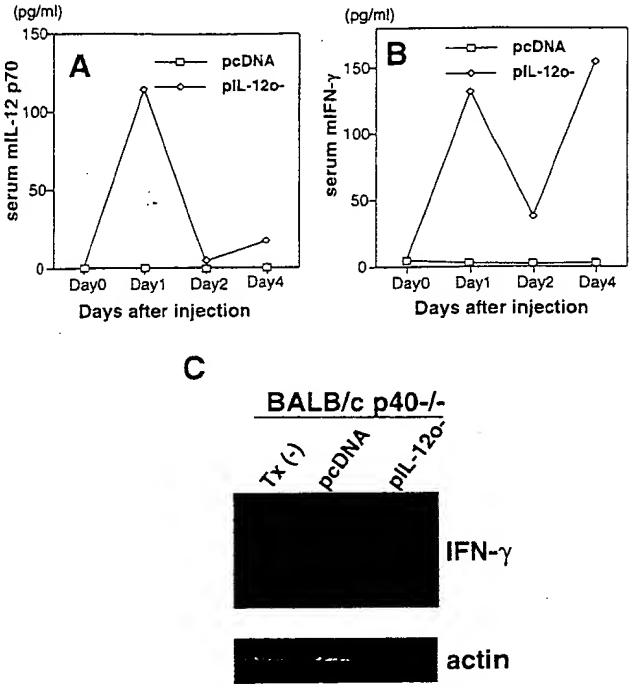

FIGURE 10. Detection of IL-12 p70 (*A*) or IFN-γ (*B*) proteins in the serum of IL-12 p40$^{-/-}$ mice. Fifty micrograms of DNA was injected once i.d., and IL-12 p70 or IFN-γ was measured in the serum by ELISA on days 1, 2, and 4. IFN-γ gene induction 24 h after plasmid injection (*C*) is shown for spleens obtained from the same mice as those used for the protein analyses in *A* and *B*.

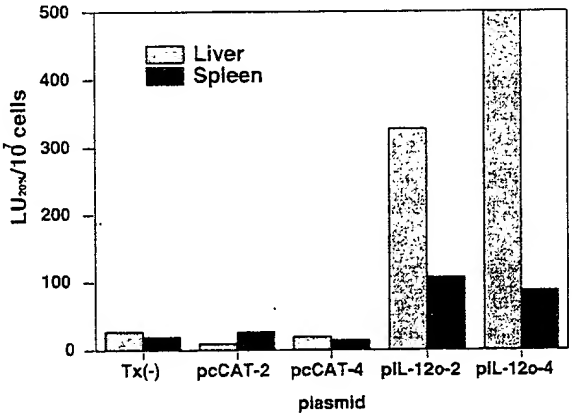

FIGURE 9. The i.v. gene delivery of pIL-12o$^-$ and pcDNA. Two hundred micrograms of DNA dissolved in PBS containing 5% glucose was injected into the tail vein of BALB/c mice. Four days later leukocytes were isolated from the liver and incubated for 4 h with ^{51}Cr-labeled YAC-1 target cells.

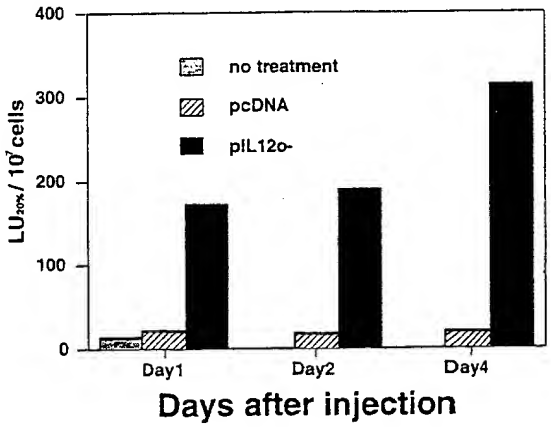

FIGURE 11. Induction of NK lytic activity induced by i.d. injection of pIL-12o$^-$ in IL-12 p40$^{-/-}$ BALB/c mice. Leukocytes isolated from the spleens of the same mice that were monitored for serum cytokines in Fig. 10 were incubated with ^{51}Cr-labeled YAC-1 cells for 4 h.

Discussion

IL-12 has proven to be a very active agent for immune modulation and for treatment of infectious and neoplastic diseases in animal models. However, much remains to be learned about the most appropriate manner in which to deliver this cytokine for maximal therapeutic benefit and with minimal toxicity. The studies presented herein focus on an approach using naked DNA to transfect the IL-12 gene into host cells in vivo. The design of the expression vectors used for our studies is complicated by the heterodimeric nature of IL-12, and we have made the assumption that a single vector, encoding both chains of the IL-12 protein, would be preferable to individual plasmids for p40 and p35. Because the p40 subunit is usually produced in large excess over the p70 dimer, but only the latter has biological activity (2), we speculated that a single plasmid encoding both genes would enhance the potential of obtaining mostly p70 and less free p40, which has been postulated to play a role in inhibiting p70 function (39, 40). Therefore, we constructed four IL-12 expression vectors based on this theory and tested these plasmids for their abilities to induce several characteristic indicators of systemic IL-12 activity. Specifically, we chose augmentation of NK activity and induction of IFN-γ gene expression and protein production as the parameters of choice for detecting systemic IL-12-induced bioactivities. We found that pIL12o$^+$, in which the p40 and p35 subunits were driven by a separate CMV promoter/enhancer and where the expression cassettes were oriented in the opposite direction in the same construct, produced the highest NK cell activity in the liver and spleen. The degree of systemic augmentation of NK activity was higher than that achieved with other plasmids, including those in which p40 and p35 were both in the same orientation but driven by individual CMV promoter/enhancers or using an IRES plasmid in which the expression cassette was in a tandem orientation. Some previous reports also suggested that the coexistence of CMV enhancer and SV40 promoter for neomycin expression decreased gene expression from the CMV enhancer (41). Therefore, in an attempt to obtain the most efficient in vivo construct possible, IL-12o$^-$, the neomycin expression cassette was removed from the pIL-12o$^+$ plasmid, and this pIL-12o$^-$ demonstrated NK activity at least comparable with that obtained using pIL-12o$^+$. In addition, pIL-12o$^-$ was superior to the pIL-12o$^+$ construct for induction of IFN-γ in vivo (data not shown). These results extend previous studies (34) that reported an augmentation of splenic NK activity by i.d. injection of IL-12 cDNA by demonstrating that the pIL-12 also can augment NK activity in the liver.

The best cellular target for in vivo transfection remains unclear. In our initial experiments we chose the i.d. route to deliver naked DNA for two reasons. First, it is technically simpler than i.m. injection, and second, the previous literature demonstrates that i.d. transferred genes are expressed more quickly than those expressed after injection into muscle. Because we also plan to contrast naked DNA delivery with protein therapy for the treatment of rapidly growing established tumors in mice, we speculate that a process that results in more rapid gene expression in vivo could be more beneficial than one where initial gene expression is more prolonged, but delayed in its onset. As shown in Fig. 2, the injected DNA was expressed mainly in the i.d. smooth muscle, while in previous studies the activity of the i.d. injected gene product was visualized predominantly in the epidermis in the human and pig skin tissue (32). Thus, there may be a preference for a particular cell type for the DNA entry depending on the nature of the construct, the genes to be expressed, or the species to be injected. By Northern blot analysis, p40 gene expression was detectable up to 7 days at the site of the pIL-12o$^-$ injection, and mRNA expression was highest at 24 h, with another peak appearing 5 days later after a single injection of plasmid. Serum levels for IL-12 after pIL-12o$^-$ injection paralleled the kinetics of this p40 gene expression, supporting the observed biphasic nature of p40 gene expression. This biphasic pattern of expression was surprising, and we speculated that the first peak represented IL-12 produced from the injected plasmid, while the second peak could be due to subsequent production of IFN-γ by T and NK cells (42) and subsequently induction of more IL-12 production by phagocytic cells (43). However, this is not the case, because the same biphasic gene expression is obtained after pIL-12o$^-$ injection into p40$^{-/-}$ mice, in which endogenous IL-12 p70 cannot be induced. Taking these three independent results (p40 Northern blotting, IL-12 and IFN-γ serum ELISAs in normal mice and p40$^{-/-}$ mice) together, therefore, we conclude that i.d. pIL-12o$^-$ injection results in a biphasic expression pattern for the transferred gene itself in vivo. Although previous studies using i.m. DNA injection revealed a gradual increase in protein expression by the transgene up to 14 days, with activity detected as long as 120 days (29, 44), we know of no reports of clear biphasic or intermittent expression. To date, the relatively short term (e.g., 1–7 days) kinetics of naked DNA in vivo have not been investigated in complete detail, and we plan to examine the mechanism for these biphasic effects.

Taking into consideration the expression data outlined above, it may not be surprising that the highest NK cell activity in liver and spleen was not observed until about 4 days after pIL-12o$^-$ injection (i.d.) even though the blood level of IL-12 was highest at about 24 h. Recent data from our laboratory have shown that a single administration of recombinant mouse IL-12 protein (0.5 μg/day) induced the highest NK activity in the liver compared with daily injection for 2–4 days (45). In these studies a decrease in NK activity after repeated administration of IL-12 was accompanied by a reduced number of NK cells. However, in the studies using the pIL-12o$^-$ no decrease in the number of NK cells in the liver was observed even 4 days after pIL-12o$^-$ injection (data not shown), suggesting a basic biological difference in the regulation of hepatic NK cells by exogenous IL-12 protein vs pIL-12o$^-$. Interestingly, we noted in our previous studies with IL-12 protein administration that the ability of IL-12 to induce recruitment of NK cells to the liver is dependent on the production of IFN-γ (45). Therefore, IFN-γ induced by IL-12 may contribute to a recruitment of NK cells to at least some sites. In addition, the IFN-γ-inducible Mig gene, which serves as another indicator of systemic effects of IL-12, also was induced. Although this expression of Mig may have no direct relevance to induction of Th1 responses, it may play some role in the IFN-γ-dependent recruitment of NK cells induced by IL-12 (45). We are currently studying the role of IFN-γ-inducible genes in IL-12-induced leukocyte recruitment.

Another intriguing finding of our study is that in p40$^{-/-}$ mice, injection of pIL-12o$^-$ induced more potent augmentation of NK activity and induced more IFN-γ than in normal mice, particularly in the second peak. The controlled production of the p40 subunit that is usually produced in large excess over the p35 subunit and can antagonize the biological effects of IL-12 p70 may explain the higher responsiveness to IL-12 translated from the expressed pIL-12o$^-$ in p40$^{-/-}$ mice.

In an effort to further optimize and understand the immunomodulatory potential of the pIL-12o$^-$, naked DNA delivery by the i.v. route also was investigated. A previous report found that the i.v. injection of naked DNA in PBS resulted in degradation within 5 min and the absence of any protein expression in various organs (46). However, in our studies i.v. injection of 200 μg of pIL-12o$^-$

effectively induced NK activity in both liver and spleen by 2 days after injection, and the augmented NK activity remained detectable at 4 days. Consistent with this observation are studies by Wang et al., who reported kallikrein gene expression in heart, lung, and liver even 3 wk after a single i.v. injection of 500 μg of DNA dissolved in PBS containing 5% glucose (47). Thus, the addition of 5% glucose, as used in our studies, may be useful for stabilizing the DNA for i.v. injection, although the mechanism involved in the stability has not been determined yet.

In vivo delivery of plasmid DNA is becoming more commonly used as a novel vaccination method (48), where i.m. or i.d. injection of Ag-coding DNA favors the effective development of Th1 responses (49), and a previous study has demonstrated that the injection of IL-12 cDNA can actually delay the growth of subsequently injected murine renal cancer cells. A recent finding also has been reported that particular unmethylated DNA sequences with CpG motifs preferentially stimulate the production of IFN-γ, IL-12, and IL-18 (50) and can activate various effector leukocyte cells, including NK cells. In the bacterial genome these sequences are often unmethylated and 20 times as common as in mammalian DNA, whose CpG motif is methylated in >80% of the cases. Thus, some mammalian DNA and, more preferentially, bacterial DNA also may target induction of a Th1 response by host leukocytes. In contrast, other approaches, such as DNA vaccination by the gene gun, preferentially induce Th2 responses (51). Although the gene gun requires 1/100th less DNA (usually 0.5–2 μg) compared with i.d. or i.m. DNA (50–200 μg) vaccination to elicit biological effects, it also is possible that the amount of DNA delivered by the gene gun technology is not sufficient to effectively trigger Th1 immune responses locally or systemically. Our experiments in normal and $p40^{-/-}$ mice demonstrate clearly that the i.d. injection of $pIL\text{-}12o^{-}$ DNA triggers a strong Th1 response that is independent of a secondary IFN-γ-induced or DNA-nonspecific production of endogenous IL-12. Thus, the i.d. delivery of $pIL\text{-}12o^{-}$ leads to effective transcription and translation of biologically active IL-12 p70 that systemically induces cytokines and host effector cell functions.

The effective induction of a Th1 response, as indicated by IFN-γ synthesis following $pIL\text{-}12o^{-}$ in our studies, may be a major advantage for the therapeutic use of IL-12 in vaccine approaches to infectious disease and cancer treatment. Ghosh et al. reported that T cells from mice bearing tumors for >1 mo gradually lose the Th1 phenotype (52), and the reversal or prevention of this effect may be important for maximizing the response to therapeutic vaccines. In addition to any systemic effects, the amount of IL-12 available at the tumor site contributes to both the type and the number of infiltrating leukocytes and the events leading to tumor regression (53). As for infection, a similar effect has been confirmed during vaccination against *Schistosoma mansoni*, in which vaccination with eggs and IL-12 prevents the subsequent pulmonary granuloma formation and tissue fibrosis that are associated with a Th2-dominated pattern of cytokine expression (54, 55). Also, as mentioned previously, IL-12 plays an important role in protecting against infection by *L. major* (24).

Overall, the IL-12 gene delivery approach described herein demonstrates the potency of appropriate DNA expression plasmids for the induction of systemic and local Th1-type responses. Such effects, in the absence of the practical limitations often observed during the use of viral vectors as gene delivery systems, suggest the considerable utility of this approach for vaccine-based prophylactic or therapeutic strategies in the treatment or prevention of infections and neoplastic diseases.

Acknowledgments

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References

1. Kobayashi, M., L. Fitz, M. Ryan, R. M. Hewick, S. C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med. 170:827.*
2. Wolf, S. F., P. A. Temple, M. Kobayashi, D. Young, M. Dicig, L. Lowe, R. Dzialo, L. Fitz, C. Ferenz, R. M. Hewick, et al. 1991. Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. *J. Immunol. 146:3074.*
3. Gately, M. K., A. G. Wolitzky, P. M. Quinn, and R. Chizzonite. 1992. Regulation of human cytolytic lymphocyte responses by interleukin-12. *Cell. Immunol. 143: 127.*
4. Trinchieri, G. 1994. Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. *Blood 84:4008.*
5. Hendrzak, J. A., and M. J. Brunda. 1995. Interleukin-12: biologic activity, therapeutic utility, and role in disease. *Lab. Invest. 72:619.*
6. Hayes, M. P., J. Wang, and M. A. Norcross. 1995. Regulation of interleukin-12 expression in human monocytes: selective priming by interferon-γ of lipopolysaccharide-inducible p35 and p40 genes. *Blood 86:646.*
7. Trinchieri, G., and P. Scott. 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions. *Res. Immunol. 146:423.*
8. Manetti, R., P. Parronchi, M. G. Giudizi, M.-P. Piccini, E. Maggi, G. Trinchieri, and S. Romagnani. 1993. Natural killer cell stimulatory factor (interleukin 12 [IL-12]). *J. Exp. Med. 177:1199.*
9. Trinchieri, G. 1993. Interleukin-12 and its role in the generation of TH1 cells. *Immunol. Today 14:335.*
10. Wigginton, J. M., D. B. Kuhns, T. C. Back, M. J. Brunda, R. H. Wiltrout, and G. W. Cox. 1996. Interleukin 12 primes macrophages for nitric oxide production in vivo and restores depressed nitric oxide production by macrophages from tumor-bearing mice: implications for the antitumor activity of interleukin 12 and/or interleukin 2. *Cancer Res. 56:1131.*
11. Voest, E. E., B. M. Kenyon, M. S. O'Reilly, G. Truitt, R. J. D'Amato, and J. Folkman. 1995. Inhibition of angiogenesis in vivo by interleukin 12. *J. Natl. Cancer Inst. 87:581.*
12. Majewski, S., M. Marczak, A. Szmurlo, S. Jablonska, and W. Bollag. 1996. Interleukin-12 inhibits angiogenesis induced by human tumor cell lines in vivo. *J. Invest. Dermatol. 106:1114.*
13. Brunda, M. J., L. Luistro, R. R. Warrier, R. B. Wright, B. R. Hubbard, M. Murphy, S. F. Wolf, and M. K. Gately. 1993. Antitumor and antimetastatic activity of interleukin 12 against murine tumors. *J. Exp. Med. 178:1223.*
14. Brunda, M. J. 1994. Interleukin-12. *J. Leukocyte Biol. 55:280.*
15. Zou, J. P., N. Yamamoto, T. Fujii, H. Takenaka, M. Kobayashi, S. H. Herrmann, S. F. Wolf, H. Fujiwara, and T. Hamaoka. 1995. Systemic administration of rIL-12 induces complete tumor regression and protective immunity: response is correlated with a striking reversal of suppressed IFN-γ production by anti-tumor T cells. *Int. Immunol. 7:1135.*
16. Wigginton, J. M., K. L. Komschlies, T. C. Back, J. L. Franco, M. J. Brunda, and R. H. Wiltrout. 1996. Administration of interleukin 12 with pulse interleukin 2 and the rapid and complete eradication of murine renal carcinoma. *J. Natl. Cancer Inst. 88:38.*
17. Uharek, L., M. Zeis, B. Glass, J. Steinmann, P. Dreger, W. Gassmann, N. Schmitz, and W. Muller-Ruchholtz. 1996. High lytic activity against human leukemia cells after activation of allogeneic NK cells by IL-12 and IL-2. *Leukemia 10:1758.*
18. Watanabe, M., K. L. McCormick, K. Volker, J. R. Ortaldo, J. M. Wigginton, M. J. Brunda, R. H. Wiltrout, and W. E. Fogler. 1997. Regulation of local host-mediated anti-tumor mechanisms by cytokines: direct and indirect effects on leukocyte recruitment and angiogenesis. *Am. J. Pathol. 150:1869.*
19. Noguchi, Y., E. C. Richards, Y. T. Chen, and L. J. Old. 1995. Influence of interleukin 12 on p53 peptide vaccination against established Meth A sarcoma. *Proc. Natl. Acad. Sci. USA 92:2219.*
20. Bramson, J. L., M. Hitt, C. L. Addison, W. J. Muller, J. Gauldie, and F. L. Graham. 1996. Direct intratumoral injection of an adenovirus expressing interleukin-12 induces regression and long-lasting immunity that is associated with highly localized expression of interleukin-12. *Hum. Gene Ther. 7:1995.*
21. Chen, L., D. Chen, E. Block, M. O'Donnell, D. W. Kufe, and S. K. Clinton. 1997. Eradication of murine bladder carcinoma by intratumor injection of a bicistronic adenoviral vector carrying cDNAs for the IL-12 heterodimer and its inhibition by the IL-12 p40 subunit homodimer. *J. Immunol. 159:351.*
22. Caruso, M., K. Pham-Nguyen, Y. L. Kwong, B. Xu, K. I. Kosai, M. Finegold, S. L. Woo, and S. H. Chen. 1996. Adenovirus-mediated interleukin-12 gene therapy for metastatic colon carcinoma. *Proc. Natl. Acad. Sci. USA 93:11302.*

23. Siders, W. M., P. W. Wright, J. A. Hixon, W. G. Alvord, T. C. Back, R. H. Wiltrout, and R. G. Fenton. 1998. T cell- and NK cell-independent inhibition of hepatic metastases by systemic administration of an IL-12-expressing recombinant adenovirus. *J. Immunol. 160:5465.*
24. Magram, J., J. Sfarra, S. Connaughton, D. Faherty, R. Warrier, D. Carvajal, C. Y. Wu, C. Stewart, U. Sarmiento, and M. K. Gately. 1996. IL-12-deficient mice are defective but not devoid of type 1 cytokine responses. *Ann. NY Acad. Sci. 795:60.*
25. Brunda, M. J., L. Luistro, R. R. Warrior, R. B. Wright, B. R. Hubbard, M. Murphy, S. F. Wolf, and M. K. Gately. 1993. Antitumor and antimetastatic activity of interleukin 12 against murine. *J. Exp. Med. 178:1223.*
26. Tannenbaum, C. S., N. Wicker, D. Armstrong, R. Tubbs, J. Finke, R. M. Bukowski, and T. A. Hamilton. 1996. Cytokine and chemokine expression in tumors of mice receiving systemic therapy with IL-12. *J. Immunol. 156:693.*
27. Cohen, J. 1995. IL-12 deaths: explanation and a puzzle. *Science 270:908.*
28. Orange, J. S., T. P. Salazar-Mather, S. M. Opal, R. L. Spencer, A. H. Miller, B. S. McEwen, and C. A. Biron. 1995. Mechanism of interleukin 12-mediated toxicities during experimental viral infections: role of tumor necrosis factor and glucocorticoids. *J. Exp. Med. 181:901.*
29. Wolff, J. A., R. W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, and P. L. Felgner. 1990. Direct gene transfer into mouse muscle in vivo. *Science 247:1465.*
30. Wolff, J. A., J. J. Ludtke, G. Acsadi, P. Williams, and A. Jani. 1992. Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Hum. Mol. Genet. 1:363.*
31. Raz, E., D. A. Carson, S. E. Parker, T. B. Parr, A. M. Abai, G. Aichinger, S. H. Gromkowski, M. Singh, D. Lew, M. A. Yankauckas, et al. 1994. Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses. *Proc. Natl. Acad. Sci. USA 91:9519.*
32. Hengge, U. R., E. F. Chan, R. A. Foster, P. S. Walker, and J. C. Vogel. 1995. Cytokine gene expression in epidermis with biological effects following injection of naked DNA. *Nat. Genet. 10:161.*
33. Hengge, U. R., P. S. Walker, and J. C. Vogel. 1996. Expression of naked DNA in human, pig, and mouse skin. *J. Clin. Invest. 97:2911.*
34. Tan, J., C. A. Newton, J. Y. Djeu, D. E. Gutsch, A. E. Chang, N. S. Yang, T. W. Klein, and Y. Hua. 1996. Injection of complementary DNA encoding interleukin-12 inhibits tumor establishment at a distant site in a murine renal carcinoma model. *Cancer Res. 56:3399.*
35. Tang, D. C., M. DeVit, and S. A. Johnston. 1992. Genetic immunization is a simple method for eliciting an immune. *Nature 356:152.*
36. Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, S. H. Dromkowski, R. R. Deck, C. M. DeWitt, A. Friedman, et al. 1993. Heterologous protection against influenza by injection of DNA encoding. *Science 259:1745.*
37. Kessler, P. D., G. M. Podsakoff, X. Chen, S. A. McQuiston, P. C. Colosi, L. A. Matelis, G. J. Kurtzman, and B. J. Byrne. 1996. Gene delivery to skeletal muscle results in sustained expression and systemic delivery of a therapeutic protein. *Proc. Natl. Acad. Sci. USA 93:14082.*
38. Magram, J., S. E. Connaughton, R. R. Warrier, D. M. Carvajal, C. Y. Wu, J. Ferrante, C. Stewart, U. Sarmiento, D. A. Faherty, and M. K. Gately. 1996. IL-12-deficient mice are defective in IFN γ production and type 1 cytokine responses. *Immunity 4:471.*
39. Tahara, H., L. Zitvogel, W. J. Storkus, H. J. R. Zeh, T. G. McKinney, R. D. Schreiber, U. Gubler, P. D. Robbins, and M. T. Lotze. 1995. Effective eradication of established murine tumors with IL-12 gene therapy using a polycistronic retroviral vector. *J. Immunol. 154:6466.*
40. Rakhmilevich, A. L., J. Turner, M. J. Ford, D. McCabe, W. H. Sun, P. M. Sondel, K. Grota, and N. S. Yang. 1996. Gene gun-mediated skin transfection with interleukin 12 gene results in regression of established primary and metastatic murine tumors. *Proc. Natl. Acad. Sci. USA 93:6291.*
41. Kobatashi, M., A. Tanaka, Y. Hayashi, and S. S. 1997. The CMV enhancer stimulates expression of foreign genes from the human EF-1α promoter. *Anal. Biochem.247:179.*
42. Chan, S. H., B. Perussia, J. W. Gupta, M. Kobayashi, M. Pospisil, H. A. Young, S. F. Wolf, D. Young, S. C. Clark, and G. Trinchieri. 1991. Induction of interferon γ production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. *J. Exp. Med. 173:869.*
43. Trinchieri, G. 1995. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu. Rev. Immunol. 13:251.*
44. Manthorpe, M., F. Cornetert-Jensen, J. Hartikka, J. Felgner, A. Rundell, M. Margalith, and V. Dwarki. 1993. Gene therapy by intramuscular injection of plasmid DNA: studies on firefly luciferase gene expression in mice. *Hum. Gene Ther. 4:419.*
45. Fogler, W. E., K. Volker, M. Watanabe, J. M. Wigginton, P. Roessler, M. J. Brunda, J. R. Ortaldo, and R. H. Wiltrout. 1998. Recruitment of hepatic NK cells by IL-12 is dependent on IFN-γ and VCAM-1 and is rapidly down-regulated by a mechanism involving T cells and expression of Fas. *J. Immunol. 161:6014.*
46. Osaka, G., K. Carey, A. Cuthbertson, P. Godowski, T. Patapoff, A. Ryan, T. Gadek, and J. Mordenti. 1996. Pharmacokinetics, tissue distribution, and expression efficiency of plasmid [^{33}P]DNA following intravenous administration of DNA/cationic lipid complexes in mice: use of a novel radionuclide approach. *J. Pharm. Sci. 85:612.*
47. Wang, C., L. Chao, and J. Chao. 1995. Direct gene delivery of human tissue kallikrein reduces blood pressure in spontaneously hypertensive rats. *J. Clin. Invest. 95:1710.*
48. Donnelly, J. J., J. B. Ulmer, J. W. Shiver, and M. A. Liu. 1997. DNA vaccines. *Annu. Rev. Immunol. 15:617.*
49. Manickan, E., R. J. Rouse, Z. Yu, W. S. Wire, and B. T. Rouse. 1995. Genetic immunization against herpes simplex virus: protection is mediated by CD4$^+$ T lymphocytes. *J. Immunol. 155:259.*
50. Roman, M., E. Martin-Orozco, J. S. Goodman, M. D. Nguyen, Y. Sato, A. Ronaghy, R. S. Kornbluth, D. D. Richman, D. A. Carson, and E. Raz. 1997. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat. Med. 3:849.*
51. Feltquate, D. M., S. Heaney, R. G. Webster, and H. L. Robinson. 1997. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *J. Immunol. 158:2278.*
52. Ghosh, P., K. L. Komschlies, M. Cippitelli, D. L. Longo, J. Subleski, J. Ye, A. Sica, H. A. Young, R. H. Wiltrout, and A. C. Ochoa. 1995. Gradual loss of T-helper 1 populations in spleen of mice during progressive tumor growth. *J. Natl. Cancer Inst. 87:1478.*
53. Colombo, M. P., M. Vagliani, F. Spreafico, M. Parenza, C. Chiodoni, C. Melani, and A. Stoppacciaro. 1996. Amount of interleukin 12 available at the tumor site is critical for tumor regression. *Cancer Res. 56:2531.*
54. Wynn, T. A., D. Jankovic, S. Hieny, A. W. Cheever, and A. Sher. 1995. IL-12 enhances vaccine-induced immunity to *Schistosoma mansoni* in mice and decreases T helper 2 cytokine expression, IgE production, and tissue eosinophilia. *J. Immunol. 154:4701.*
55. Wynn, T. A., A. W. Cheever, D. Jankovic, R. W. Poindexter, P. Caspar, F. A. Lewis, and A. Sher. 1995. An IL-12-based vaccination method for preventing fibrosis induced by schistosome infection. *Nature 376:594.*